Investigating the Potential of Biosurfactants in the Control of Tooth Infections

A thesis submitted to Cardiff University in fulfilment of the requirements for the degree of

Doctor of Philosophy (PhD)

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Dedication

to IRAQ in general, NINAVA in particular

I dedicate this thesis

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First and foremost, I praise ALLAH, the almighty for granting me the opportunity, capability and providing me with patience to complete this Thesis. Pursuing my PhD has been an incredible challenge especially when I suddenly found myself alone raising three children away from my family whom themselves were in danger. This journey would not have been possible to be completed successfully without the help and support of those around me.

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Zahraa Hashim, Melanie J Wilson, Jean-Yves Maillard, Rachel Waddington

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Zahraa Hashim, Daniel J Morse, Jean-Yves Maillard, Melanie J Wilson, Rachel J Waddington

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Zahraa Hashim, Jean-Yves Maillard, Melanie J Wilson, Rachel J Waddington

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Zahraa Hashim, Jean-Yves Maillard, Melanie J Wilson, Rachel J Waddington

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Zahraa Hashim, Jean-Yves Maillard, Melanie J Wilson, Rachel J Waddington

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Zahraa Hashim, Jean-Yves Maillard, Melanie J Wilson, Rachel J Waddington

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"Assessment of Biosurfactants from Lactobacillus plantarum to Fight Endodontic Infection"

Zahraa Hashim, Jean-Yves Maillard, Melanie J Wilson, Rachel J Waddington

Abstract

A shift from the "doomed" organ concept of an exposed pulp to the concept of hope and retrieval has been recently adopted with the introduction of vital pulp therapy. Microbial contamination of the capped pulp is the main cause of therapy failure and is a fundamental challenge of contemporary endodontics. Biosurfactants are tensioactive microbe-derived molecules with a potential antimicrobial/antiadhesive activity. This project aimed to investigate the role of biosurfactants, specifically probiotic-derived ones and a commercial-sourced rhamnolipid (Sigma Aldrich), as novel endodontic antimicrobial therapies that can aid in reducing the incidence of infection and endodontic failure.

Of five probiotic strains studied, *Lactobacillus plantarum* demonstrated significant antimicrobial activity against three *Streptococcus anginosus* group members; *S. anginosus, S. constellatus* and *S. intermedius* and against *Enterococcus faecalis*. A cell-bound biosurfactant (*Lp*-BS) was successfully extracted from the potential probiotic *L. plantarum.* Biochemical characterisation identified *Lp*-BS as a glycoprotein molecule that was capable of reducing surface tension and emulsifying hydrocarbons. For the endodontic pathogens studied, *Lp*-BS demonstrated no antibacterial activity up to concentrations of 50 mg/ml, but displayed significant antiadhesive potential at 20 mg/mL. Proteomic analysis revealed a rich heterogeneous proteinaceous nature of *Lp*-BS mixture and importantly identified three adhesin-like proteins raising the possibility for applications in pathogenic bacterial adhesion interference. Ten mg/ml *Lp*-BS induced high levels of apoptosis in pulpal fibroblasts at 24 h, resulting in significant reduction in cell number. No significant differences were reported in cell counts or histology of pulp tissue treated with 0.625-10 mg/mL *Lp*-BS in comparison to the controls, over a 24 h time period. Subsequent incubation with 10 mg/mL for 48 h revealed obvious pulp tissue toxicity. Partial purification of *Lp*-BS by means of size-exclusion chromatography resulted in five elution pools with the first fraction determined to retain the biosurfactant activity; this fraction can be further investigated for antimicrobial/antiadhesive effects. Rhamnolipid demonstrated substantial antimicrobial effect with minimum inhibitory concentrations determined to be 0.097 mg/mL against *S. anginosus*, 0.048 mg/mL against *S. constellatus* and *S. intermedius* and 50 mg/mL against *E. faecalis*. Rhamnolipid also reduced attachment of *S. anginosus* and *S. intermedius* to an abiotic surface. Of importance, rhamnolipid was shown to tolerate serum concentrations ≤ 5 % v/v. However, above this concentration inactivation of rhamnolipid effect was demonstrated. Application of rhamnolipid to rodent *ex-vivo* tooth model infected with *S. anginosus* and *S. constellatus* demonstrated significant antimicrobial activity and a mild immune response of pulp tissue in response to treatment was documented.

This work provides the foundation for further investigation on *Lp*-BS adhesin-like proteins as a novel endodontic antiadhesive therapy. Furthermore, the cost-effective rhamnolipid with its antimicrobial and immune-modulatory effect covers two fundamental requirements of pulp tissue repair and therefore, opens future prospects for novel biosurfactants application in pulp therapy.

List of Abbreviations

AS Aggregation substance

B2M beta-2-microglobulin

BCA Bicinchoninic Acid Protein Assay

BHI Brain heart infusion

CFU Colony forming unit

CHO Carbohydrate

CLSM Confocal laser scanning microscopy

CMC Critical micelle concentration

CO_2 Carbon dioxide

CSP Competence-stimulating signalling peptide

DMEM Dulbecco's Modified Eagle's Medium

DMSO Dimethyl Sulfoxide

DNA Deoxyribonucleic acid

DW Distilled water

E. coli *Escherichia coli*

E. faecalis *Enterococcus faecalis*

ECM Extracellular matrix

EF Elongation factor

EI Emulsifying index

ELISA Enzyme Linked-Immunosorbent Assay

FAA Fastidious anaerobe agar

FBS Foetal bovine serum

FITC Fluorescein isothiocyanate

FPLC Fast protein liquid chromatography

ftf fructosyltransferase

FT-IR Fourier transform infrared spectroscopy

GAG Glycosaminoglycan

GAPDH Glyceraldehyde-3-phosphate dehydrogenase

GRAS Generally regarded as safe

gtf glucosyltransferases

H Hour

H&E Haemotoxylin and eosin

HPLC High performance liquid chromatography

IL Interleukin

***L*.** *Lactobacillus*

LAB Lactic acid bacteria

LC–MS Liquid chromatography–mass spectrometry

LDH Lactate dehydrogenase

LPS Lipopolysaccharide

M Molar

MEM Modified Minimum Essential Medium

MIC Minimum inhibitory concentration

mRNA Messenger ribonucleic acid

MRS De Man, Rogosa and Sharpe

MSCRAMMs Microbial surface components recognising adhesive matrix molecules

MTA Mineral trioxide aggregate

MWCO Molecular weight cut-off

NaCl Sodium chloride

NaOH Sodium hydroxide

NK Natural killer

NMR Nuclear magnetic resonance

OD Optical density

PAGE Polyacrylamide gel electrophoresis

PBS Phosphate-buffered saline

PCR Polymerase chain reaction

PMMA Poly (methyl-methacrylate)

qPCR Quantitative polymerase chain reaction

QS Quorum sensing

rRNA Ribosomal ribonucleic acid

RT Reverse transcription

SAG *Streptococcus anginosus* group

SD Standard deviation

SDS Sodium dodecyl sulphate

SEM Scanning electron microscopy

SMG *Streptococcus milleri* group

ST Surface tension

Td Doubling time

TGF Transforming growth factor

TLC thin layer chromatography

TLR Toll-like receptor

TMB tetramethylbenzidine

TNF Tumour necrosis factor

TUNEL Terminal deoxynucleotidyl transferase (TdT) dUTP Nick End Labeling

VPT Vital pulp therapy

Contents

List of figures

List of tables

1 Chapter 1 Introduction

1.1 General introduction

Endodontics is defined as the field of dentistry that is concerned with the morphology, physiology, and pathology of human dental pulp and periradicular tissues (Sundqvist 1976). Pulpitis mainly results as a sequel of untreated dental caries of the enamel or dentine tissue, causing exposure of the internal dental tissue to the oral cavity which in turn can allow for the invasion of bacteria into the dental pulp. As the lesion progresses it can stimulate an inflammatory response in the pulp (Ali and Mulay 2015). Pulpitis can be classified clinically as either reversible or irreversible. The distinction between the two conditions is important since the former can be successfully reversed and allow healing either with or without pulp exposure while the latter leads to pulp necrosis (Piattelli and Traini 2007). Infected teeth that have been diagnosed clinically as having irreversible pulpitis are mostly treated by root canal therapy which basically involves removal of the devitalised pulp tissue and filling the root canal with inert materials. Current endodontic treatment strategies are meant to eradicate bacteria from the root canal system by mechanical (Byström and Sundqvist 1981) and chemical means (Kim 2014). It has been found that, besides their cost and time consumption, the outcome of these strategies vary considerably with a success rate ranging from 30 to 98 % with the presence of persisting or secondary intraradicular infection accounting for the most common reason for failure (Siqueira 2001; Friedman and Mor 2004; Nair 2006). Furthermore, chemical treatments are reported to be associated with deleterious influences on the physical properties of dentine (Grigoratos *et al*. 2001; Andreasen *et al*. 2002) Therefore a shift towards biological alternatives in treating root canal without such deleterious effects on the dentine would be beneficial. Moreover, loss of vital pulp tissue excludes the possibility of reparative and regenerative dentinogenesis which is especially important in young permanent teeth where root formation has not been completed resulting in defected crown/root ratio and thin dentin wall predisposing the tooth to fracture. Therefore, maintaining pulp vitality appears to be important. In contemporary endodontics, vital pulp therapy (VPT) has been considered as an ultra-conservative treatment modality which is a new but challenging procedure to preserve a vital pulp (Asgary *et al.* 2014). This procedure, based on the level of pulp preservation, basically involves stepwise excavation, indirect pulp capping (IDPC), direct pulp capping (DPC), miniature pulpotomy (MP), partial pulpotomy and

coronal/complete pulpotomy (CP). Many factors affect the success rate of such vital therapy including *a.*) the remaining pulp being either non-inflamed or capable of healing; *b.*) proper control of haemorrhage; *c.*) application of a biocompatible/regenerative capping material; and *d.)* presence of a bacterial-tight seal (Witherspoon 2008; Aguilar and Linsuwanont 2011; Asgary and Ahmadyar 2013). The success of VPT has been proven on traumatically exposed pulps (Cvek 1978; Swift *et al.* 2003; Trope 2008). However, VPT on cariously exposed pulps has not been shown to be as successful (Cvek 1978; Barthel *et al.* 2000). The success rate has been reported to reduce over time (Amini and Parirokh 2008; Dammaschke *et al.* 2010; Hargreaves and Berman 2015). Microbial contamination seems to be the key factor in the outcome of VPT. Unfavourable outcomes can be caused by infection due to either remaining bacteria or due to the introduction of new bacteria (Barthel *et al.* 2000; Al-Hiyasat *et al.* 2006; Dammaschke *et al.* 2010; Neelakantan *et al.* 2012). The currently available VPTs are relatively expensive (Çalışkan and Güneri 2017) and not widely used in clinical practice even in developed countries (Chin *et al.* 2016). This highlights the need for more effective antimicrobial agents for pulp therapy particularly with the knowledge that the prescription of antibiotics is ineffective both before and after endodontic infection and systemic antibiotic administration has no effect in relieving painful pulpitis or resolving periradicular symptom (Aminoshariae and Kulild 2015).

A considerable variety of diverse microbial species have been recognised as playing a role in pulpitis, with the major species responsible for the virulence shifting as the infection progresses (Featherstone 2000). This study focused on two significant type of oral pathogens; *Streptococcus anginosus* group (SAG) and *Enterococcus faecalis (E. faecalis)*. *Streptococcus anginosus* group includes *S. anginosus, S. intermedius* and *S. constellatus* and is commonly considered as part of the body's commensal microflora. Nonetheless these typically facultative bacteria have been isolated as predominant components of a number of orofacial infections including dentoalveolar and periodontal abscesses, are often isolated in pure culture and are frequently associated with failed root canal treatment (Jacobs *et al*. 2003; Okayama *et al*. 2005; Ledezma-Rasillo *et al*. 2010). Whilst present in low numbers in dental plaque, it has been proposed that members of SAG can present early in the pathogenic process and may actually initiate infection, thereafter preparing the environment for

subsequent colonisation by anaerobic species (Aderhold *et al.* 1981; Gossling 1988; Shinzato and Saito 1994; Nagashima *et al.* 1999; Love and Jenkinson 2002).

Based on this background, the development of new therapeutic treatments that can control infection and prevent re-infection, thus extending the life of the capped pulp has been an increasing need.

Biosurfactants, amphiphilic tension-active agents of biological origin, have found themselves a niche in commercial markets due to their unique properties such as antiadhesive, antimicrobial, antitumor and immune-modulatory (Santos *et al.* 2016). In addition they have advantages over their synthetic counterparts in being eco-friendly, biodegradable and stable at pH and salinity extremities. Their applications have been growing recently to be used in various fields of industry, bioremediation and oil processing, biomedical and pharmaceuticals (Akbari *et al.* 2018). A wide range of bacterial species have been reported to produce biosurfactants including pathogenic and probiotic bacteria. Antimicrobial/antiadhesive effects of biosurfactants have been established in some studies against nosocomial pathogens on inanimate surfaces such as silicone rubber used in prostheses (Falagas 2009; Santos *et al.* 2016), however there are no apparent studies investigating the potential for biosurfactants in preventing the establishment of pathogenic biofilms on mammalian tissues associated with endodontic infections. The work presented in this thesis will aim to achieve this, progressing from a number of *in-vitro* assays into utilizing an infective co-culture *ex-vivo* tooth model previously developed with our research group.

The project aims to assess biosurfactants as an effective antimicrobial dental agent. Ultimately, an ideal endodontic product material should aim to:

1- Sterilise the pulp during pulp therapy and reduce the incidence of failure due to re-infection;
2- Reduce the need for further and more complex treatment or tooth extraction;
3- Reduce the need to use systemic antibiotics, which are often ineffective against the polymicrobial infections found in the tooth and impact negatively on the global problem of antimicrobial resistance;
4- Employ green chemistry in endodontics.

1.2 Tooth anatomy

1.2.1 Enamel

Mature tooth enamel (Figure 1.1) is acellular, has no blood circulation and, in contrast to dentine, does not regenerate itself. It is the most mineralised tissue of the body containing 96 % minerals which makes it able to absorb blunt heavy masticatory forces (Robinson *et al*. 1998; Fincham *et al.* 1999). The enamel surface contains an adsorbed pellicle which is an acellular layer of salivary and other macromolecules and it has a protective role (Siqueira *et al*. 2007). However, bacteria can interact with these pellicle molecules via a lock and key mechanism and may establish a biofilm in the form of plaque (Siqueira *et al.* 2012). Acid produced by plaque dwelling bacteria can affect the enamel. The high water content of the organic enamel matrix makes it permeable to hydrogen ions (Cawson and Odell 2008). As a consequence, enamel erodes leading to the development of dental caries. The latter considered as one of the most prevalent chronic diseases of humans all over the world (Selwitz *et al.* 2007).

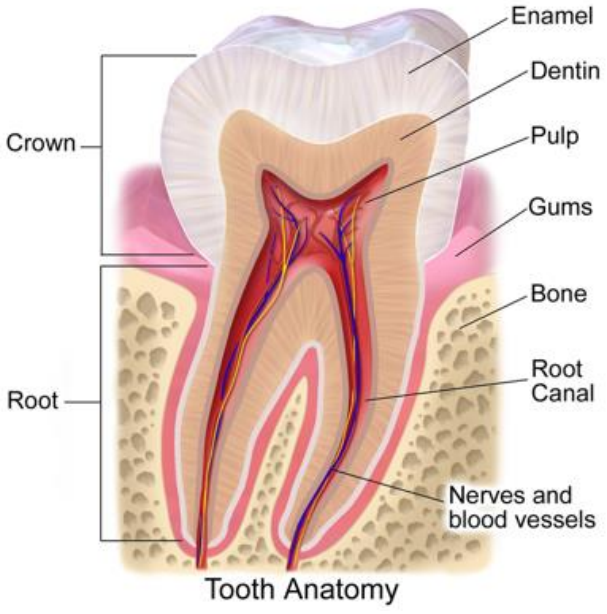


Figure 1.1 **Tooth anatomy, cross section.** (Blausen 2014)

1.2.2 Dentine

Dentine is a mineralised connective tissue of the teeth which is covered by the enamel on the crown and cementum on the root and surrounds the entire pulp (Figure 1.1) (Nanci 2007). Odontoblasts produce dentine and remain in place and active as a constant source for dentine synthesis (dentinogenesis) for the tooth lifespan (Couve 1986; Sloan *et al.* 1998; Sloan and Waddington 2009). These cells initially secrete a matrix (predentine) consisting of collagenous (primarily collagen type I) and non-collagenous proteins. Mineral hydroxyapatite constitutes 45 % of the dentine weight, while 33 % is organic material (largely type I collagen), and 22 % is fluid which is similar to plasma and contains albumin, fibrinogen and immunoglobulin G (IgG) (Knutsson *et al.* 1994; Marshall *et al.* 1997). Rapid dentine production occurs during tooth development (primary dentinogenesis). After tooth eruption, secondary dentinogenesis occurs and is characterised by a lower rate of deposition and a different gene expression profile, including the up-regulation of matrix proteins osteocalcin and dentine matrix acidic phosphoprotein-1 (DMP-1) and down-regulation of collagen type I and dentine sialophosphoprotein (DSPP), amongst others (Simon *et al.* 2009).

Dentine is characterised by being permeable due to the presence of closely packed channels, known as dentinal tubules, traversing its entire thickness and containing the cytoplasmic projections of odontoblast cells which enable the dentine to respond to external stimuli. Other than dynamic reaction to different stimuli, this tubular nature of dentine is considered of particular significance to infection as it permits bacteria and their products to diffuse through the dentine to the pulp, where they can provoke an immune reaction (Love and Jenkinson 2002). The protein-rich nature (particularly collagen) of both dentine and pulp allows for the attachment of bacteria possessing collagen-binding proteins, such as some *Streptococcus* species (Ackermans *et al.* 1981; Avilés-Reyes *et al.* 2017). However, pathogens and their products can escape these defences and further invade into dentinal tubules causing destruction of the odontoblasts which stimulates a further immune response in the pulp.

1.2.3 Pulp

The pulp is an unmineralised connective tissue that resides in the centre of the tooth in a space known as the pulpal chamber surrounded by the dentine. Odontoblasts, which are the pulp's specialised cells, and undifferentiated mesenchymal pluripotent progenitor cells,

which may differentiate into dentine-forming cells upon stimulation, are capable of forming dentine throughout life (Yu and Abbott 2007; Sloan and Waddington 2009). Beside their role in dentine formation, odontoblasts may help as sensory transmitters (Matthews *et al.* 1996). These cells are proposed to communicate with each other through the presence of tight, adhering and gap junctions. Gap junctions are present between and among odontoblasts and nerve fibres (Ushiyama 1989) and can offer a passageway of low electrical resistance between and among the odontoblasts and nerve fibres. The dental pulp is a unique tissue, because it does not form hard tissue spontaneously, but possesses the ability to mineralise gradually due to the aging process or to secrete reparative dentine after pulp exposure (Decup *et al.* 2000). For initial recognition and subsequent processing of antigens, the pulp is armed with cellular components of the immune system (Jontell *et al*. 1988; Jontell and Bergenholtz 1991; Jontell *et al*. 1998). The majority of these immune cells are peripheral T-cells (helper/inducer and cytotoxic/suppressor). Dendritic cells, the antigens presenting cells, are located in the odontoblastic layer (Okiji *et al.* 1997). In the more central region of the pulp are the macrophages which serve also as antigen-presenting cells (Jontell *et al.* 1988). Immune B-cells can present in healthy pulp in a small number and dramatically increase during infection (Farges *et al.* 2015). The pulp is also a sensory tissue registering different impulses such as thermal changes, mechanical distortion or trauma as a common sensation which pain (Holland 1994). Extracellular matrix (ECM) consisting mainly of collagens type I and III and proteoglycans such as decorin, biglycan, and versican, is secreted by fibroblasts within the pulp tissue (Shuttleworth *et al.* 1980; Goldberg and Smith 2004). This ECM provides support for the fibroblasts as well as acting as mediator of many of the cellular interaction. Additionally, the matrix can serve as a source of nutrients for the invading bacteria, which can adhere to the collagen component of the matrix. These collectively make the composition of the matrix a significant theme to be considered in studying pulpal disease.

1.2.4 Dentine-pulp complex

Functionally, physically and developmentally, the dentine and the pulp are related and can be considered together as dentine-pulp complex. They both originate from the mesenchymal cells and physically interact. The functional integrity of the dentine-pulp complex is elucidated during infection of the dentine as the odontoblasts act as the pulp's first line of defence, with processes that line the dentinal tubules able to sense the presence of bacteria and trigger an

immune response from the pulp before the bacteria have reached the pulpal chamber. The pulp dynamically responds as a functional unit by initiating inflammation and at the same time by forming reparative dentine by pulpal odontoblast-like cells as a defensive mechanism (Arnold *et al.* 2001).

1.2.4.1 Tertiary dentinogenesis

External influence such as injury or infection of the tooth including dental caries exposes the dentine-pulp complex to the oral cavity making it vulnerable to further infection and tissue damage. The pulp reacts by producing new dentine termed tertiary dentine (tertiary dentinogenesis). Such dentine can be produced with regular tubular matrix similar to primary and secondary dentine or dysplastic atubular dentine (Goldberg 2014). Biologically, tertiary dentine can be either reactionary or reparative (Smith *et al.* 2003) depending on the severity of the external stimuli. Mild stimuli cause up-regulation of the primary odontoblasts resulting in the production of reactionary dentine. When there is severe damage to the primary odontoblast cells by strong stimuli, newly differentiated odontoblast-like cells secret tertiary dentine, referred to as reparative dentine (Kuttler 1959; Lesot *et al.* 1993; Decup *et al.* 2000).

TGF-beta/s are expressed by odontoblasts leading to sequestration of some isoforms within the dentine matrix (Vaahtokari *et al.* 1991). TGF-beta/s have been implicated in reactionary dentinogenesis (Lesot *et al.* 1994; Smith *et al.* 1995). Local stimulation of the odontoblasts-secretion of extracellular matrix has been shown to be induced by TGF-beta1 and beta3 isoforms resulting in a local reactionary dentinogenesis up-regulation reflected by the increase in predentine thickness (Sloan and Smith 1999). TGF-beta2 has been shown to exert little effect on the odontoblasts and has not been detected in the human teeth dentine matrix (Cassidy *et al.* 1997). Its role has been suggested to involve in the tooth size and shape (Chai *et al.* 1994). It has been suggested that reactionary dentinogenesis is mediated by similar processes to primary dentinogenesis. Simon et al. (2010) stimulated odontoblast-like MDPC-23 cells with heat inactivated *Streptococcus mutans* and with growth factors known to stimulate reactionary dentinogenesis (TGF-beta1, TNF-alpha, and adrenomedullin). In these studies, activation of the p38 mitogen-activated protein kinase (MAPK) pathway was observed, which is also active during primary (but not secondary) dentinogenesis (Simon *et al.* 2010).

Reparative dentinogenesis, from a biological point of view, is a more complex process than reactionary dentinogenesis. It involves the recruitment of the progenitor cells from the dental pulp tissue. These cells then differentiate into odontoblast-like cells before they are able to secrete the reparative dentine matrix. These processes are mediated by a host of signalling molecules, including members of the TGF-beta family and bone morphogenic proteins (BMPs) (Kim 2017). The newly produced dentine should, ideally, be similar to tubular dentine where the new layer of odontoblasts can be adsorbed (Yoshiba *et al.* 1996). However, the hard tissue formation is variable between regular tubular dentine to a dysplastic atubular dentine. The objective of the tertiary dentinogenesis, whether reactionary or reparative, is the ultimate formation of a physical barrier against the insulting stimulus. If this fails, the pulp cells respond to this in the form of inflammatory reaction.

1.3 Pulpal inflammation

Inflammation of the pulp tissue is called pulpitis and can result as a response to exposure to different irritants such as microorganisms from carious lesions or their toxic by-products that diffuse through dentinal tubules (Love and Jenkinson 2002). As the tooth has a unique structure of the soft pulp being enclosed by hard tissues of the dentine, cementum and enamel, the pulpal inflammatory response is also unique when compared to other tissues. In the early pulp response to caries, the pulp is infiltrated locally, at sites beneath the offending carious injury, with inflammatory cells and mediators of the non-specific innate immunity, which are recruited through blood vessels of the pulp chamber. Polymorphonuclear (PMN) leukocytes and monocytes infiltrate the site of exposure after being stimulated by pathogen-associated molecules (such as components of the bacterial cell wall, flagellin, peptidoglycans and lipoproteins) forming an area of liquefaction necrosis (Cooper *et al.* 2010). As stated earlier (Section 1.2.4.1), dentine matrix proteins such as TGF-beta isoforms are also involved in triggering the inflammatory immune response within the pulp tissue (Lara *et al*. 2003; Abd-Elmeguid *et al*. 2012). The intensity of inflammation and subsequent development of necrosis depends on the virulence of the offending pathogen and on how efficient the host response is. If the pulp is unable to eliminate the damaging irritants, the infection persists and more extensive damage is perused.

The inflammatory orchestra, namely cell to cell communication, is coordinated by the secretion of protein molecules known as cytokines. The most important and extensively

investigated of cytokines are the interleukins (IL), tumour necrosis factors (TNF) and interferons (IFN). Over production of pro-inflammatory cytokines such as TNF-alpha, IFN-γ, IL-1beta, IL-6, IL-8, and IL-18 is found to be a characteristic of the pulp inflammatory response to carious lesion. It has been shown also that the anti-inflammatory IL-10 cytokine, which is important in the development of regulatory T cells (Tregs) that controls host immune response to pathogens is up-regulated (Farges *et al*. 2011; Elsalhy *et al*. 2013). Furthermore, it has been recently advocated that odontoblasts play a role in early dental pulp immune reaction to carious pathogens. For instance, engagement of Toll-like receptor (TLR)-2, a pattern recognition molecule of odontoblast-like cells with lipoteichoic acid (LTA), a cell wall component of Gram-positive bacteria stimulated odontoblast-like cells to produce pro- (IL-6, IL-8) and anti-inflammatory (IL-10) cytokines (Farges *et al*. 2011). Pulpal inflammation has also been reported to involve the production of the anti-inflammatory nitric oxide (NO), its role has been proposed to involve promotion of odontoblast-like cell differentiation and formation of reparative dentine (Mei *et al*. 2007; Yasuhara *et al*. 2007). It has been suggested that the pathogen recognition receptors (PRRs) family of dental pulp innate immunity plays a key role in the first-line defense. Understanding of PRRs is critical in regards to pulp immunomodulation and development of new therapeutic agents in the field of endodontics (Jang *et al.* 2015).

Bacterial lipopolysaccharide (LPS) is a major component of the Gram negative bacterial cell wall. LPS plays a crucial role in the dentine-pulp immune response and has been shown to persist in the dentine even after disinfection of the tooth (Vianna *et al.* 2007). It can induce the secretion of pro-inflammatory cytokines from the pulpal cells, including TNF-alpha, IL-6 and IL-8 (Nagaoka *et al.* 1996; Fouad and Acosta 2001). Lipopolysaccharide-binding protein (LBP), an acute-phase protein known to temper pro-inflammatory cytokine produced by bacteria-activated macrophages, has recently received special attention (Schumann 2011; Lee *et al*. 2012). Carrouel and colleagues (2013) studied the synthesis and accumulation of LBP in bacteria-challenged inflamed pulp. They concluded that LBP neutralises bacterial components before they reach or attach to pulp cells, accordingly restraining activation of the pulpal inflammatory/immune responses (Carrouel *et al.* 2013). Moreover, a study conducted by Ko et al. (2015) documented that human telomerase-derived peptide (GV1001) stimulated by LPS from *P. gingivalis* was found to be able to enter the pulp cell causing down regulation

of LPS-induced inflammatory process. This study emphasised the potential of GV1001 as an anti-inflammatory therapeutic target in vital pulp procedure as well as in the field of regenerative and tissue engineering.

Triggering of the inflammatory cascades within the pulp may progress to cause pain and dentine resorption through neurogenic inflammation and hard tissue remodelling (Love and Jenkinson 2002; Baik *et al*. 2008; Choi *et al*. 2009) and, if left untreated, bacterial invasion may result in irreversible chronic pulp inflammation, most often after a long phase of chronic inflammation. Subsequently, pulp necrosis, infection of the root canal system, and periapical disease may occur (Heyeraas and Berggreen 1999; Love and Jenkinson 2002). Consequently, it is of potential importance to understand thoroughly the pulpal inflammatory process especially in the field of immunotherapeutic agent development and appropriate dental procedures.

In contrast, a low-grade level of inflammation may be of a value for the dental repair processes (Farges *et al.* 2015). For instance, Paula-Silva and her team (2009) have reported that TNF-alpha may endorse the differentiation of pulp cells towards an odontoblastic phenotype. These TNF-alpha challenged pulp cells also demonstrated ability to induce mineralisation and increased expression of phosphoprotein and sialoprotein of the dentine (Paula-Silva *et al.* 2009).

1.4 Oral microbiology

The oral cavity is inhabited by a diverse range of microorganisms. It is estimated that the oral cavity may harbour up to 1000 different microbial species, whilst approximately 50 species are isolated per individual site. Many species are unique to this niche as they have developed a distinct specificity for oral colonisation (Aas *et al.* 2005; Dewhirst *et al.* 2010; Griffen *et al.* 2012; Abusleme *et al.* 2013). These microbial communities are regulated through sophisticated signalling systems and by host and environmental factors and are considered as a major driver of homeostasis or dysbiosis and ultimately health or disease. Mostly a homeostatic balance is found between the host and microbial communities, and the occupant microbiota is thought to contest with and exclude exogenous pathogens as a component of ecosystem stability, as well as contribute to normal tissue and immune system development (Kamada, Chen, *et al.* 2013; Kamada, Seo, *et al.* 2013). However, imbalance in this

homeostasis by environmental triggers gives opportunities for some of the oral microbes to transition from commensal relationship into pathogenic direct precursor of diseases such as dental caries and periodontitis, two of the most prevalent microbially induced disorders worldwide (Lamont *et al.* 2018).

1.4.1 Microbiology of dental caries and pulpal infection

Caries is considered as a principal global health issue and is the most widespread noncommunicable disease (World Health Organization 2013). It involves demineralisation of enamel and/or dentine by bacterial acid. The bacteria metabolise fermentable carbohydrate especially free sugar to produce acid, lowering the pH of the plaque to a critical value (< 5.5) causing dissolution of the hydroxyapatite of the tooth (Cawson and Odell 2008). The aetiology of dental caries has been described by three major hypothesis: specific plaque hypothesis, the nonspecific plaque hypothesis, and the ecological plaque hypothesis. From its name, the specific plaque hypothesis has proposed that only a few specific species, such as *S. mutans* and *S. sobrinus*, are actively involved in the disease (Loesche 1992). In contrast, the nonspecific plaque hypothesis upholds that caries is the consequence of the overall activity of the total plaque microflora, which is comprised of many bacterial species (Theilade 1986). Lastly, the ecological plaque hypothesis suggests that caries is an outcome of a shifting change in the balance of the resident microflora driven by changes in local environmental circumstances (Marsh 1994). Advances in molecular techniques allow for the identification of more bacterial species that were previously detectable by culturable means alone, thus expanding the range of bacterial species that may be involved in dental caries. Differences in bacterial profiles between primary and secondary teeth, as well as those of a number of health-associated and disease-associated species, were discovered using techniques based on extensive checkerboard DNA hybridisation analyses. Although the Gram-positive *S. mutans* has been long recognised as a major etiological agent of dental caries, it has been found that bacterial profiles alter with disease states and differ between primary and secondary dentitions. Bacterial genera other than *S. mutans*, e.g., species of the genera *Veillonella*, *Lactobacillus*, *Bifidobacterium*, and *Propionibacterium*, *Actinomyces* spp., and *Atopobium* spp., have also been identified using molecular techniques to likely play a role in disease progression (Aas *et al.* 2008). Moreover, in a study that compared the bacterial species in childhood teeth with and without caries, some genera, such as *S. sanguinis*, were

associated with health, while others, such as *S. mutans*, other *Streptococcus* spp., *Veillonella* spp., *Actinomyces* spp., *Bifidobacterium* spp., and *Lactobacillus fermentum*, were associated with caries (Becker *et al.* 2002). A diverse bacterial community was also reported for species associated with the middle and advancing front of dental caries including *S. mutans*, *Lactobacillus* spp., *Rothia dentocariosa*, and *Propionibacterium* spp. (Munson *et al.* 2004). Van der Hoeven et al. (1978) observed a reduction in caries and lower plaque lactate in rats infected with *Veillonella alcalescens* and *S. mutans* compared with *S. mutans* alone. This was proposed to be due to the ability of the former species to utilise lactic acid into a weaker acid thus reduce its damaging effect (van der Hoeven *et al.* 1978).

Even with the reported prevalence of *S. mutans* and *Lactobacillus* species in dental caries, Boyar et al. (1989) found examples of carious lesions with no evidence of these species present in extracted teeth that were banded to allow plaque growth *ex-vivo*, suggesting that these species are not always needed to start caries formation (Boyar *et al.* 1989). Strong associations were revealed for the anaerobes *Micromonas micros* (now named *Parvimonas micra*) and *Porphyromonas endodontalis* (detected by real-time polymerase chain reaction; PCR) and the development of inflammatory degeneration of pulp tissue (Martin *et al.* 2002). *P. micra* isolated from deep dentinal caries associated with pulp exposure was found associated with symptoms of throbbing pain in irreversible pulpitis (Rôças *et al.* 2015) and some strains have been shown to produce collagenase, which could account for its destructive nature in the collagen-rich pulp tissue (Ota-Tsuzuki and Alves Mayer 2010). Although enamel is the hardest tissue of the body, its porosity from demineralisation makes it more permeable to acid and pathogens. The bacteria can then diffuse into the relatively less mineralised dentino-enamel junction. The carious lesion can then spread laterally and pulpally through the dentinal tubules of the demineralised dentine causing cavity. Cavitation exposes the pulp-dentine complex to a range of oral microbes present in saliva and plaque. The dentine reacts to such bacterial insult by the formation of peritubular sclerotic dentine to block the dentinal tubules or through the formation of tertiary dentine based on the rate of progression and the type of stimuli to the pulp tissue as previously explained earlier (Section 1.2.4.1). If this fails, the carious lesion progresses changing the environment conditions. This is due to the reduction of carbohydrate, increase in pH and oxygen reduction. The changes in the environment appears to cause a shift in the microbiota from those

involved in the initiation of infection to those associated with progression. For example, microaerophilic opportunistic bacteria such as *S. anginosus* survive successfully in such a niche (Massey *et al.* 1993; Marsh 2010).

1.4.2 Pulp infection

There are many ways for microorganisms to reach and infect the pulp tissue (Estrela 2009): 1- Dentinal tubules: especially after a deep carious lesion or during dental procedures. It has been shown that the bacteria can cross to the pulp when the distance between the base of the carious lesion where the bacteria reside and the pulp is 0.2 µm (Singh 2016); 2- Cavitation: trauma such as coronal fracture breaks the physical protective shield and exposes the pulp to the septic oral cavity; 3- Periodontal membrane: bacteria can reach pulp chamber from the gingival sulcus through the periodontal membrane via the lateral channel or the apical foramen. This can occur as a result of the migration of epithelial insertion to the establishment of periodontal pockets. 4- Defective restoration: bacteria can get access to the pulp in case the temporary seal is broken or if the tooth structure fractures before final restoration, or if the final restoration is inadequate. Once the root canal chamber is infected coronally, infection can progress apically until bacteria or their products get close to the periapical tissue initiating inflammatory apical periodontitis (Torabinejad *et al.* 1990). Bacteria can approach the pulp also from the blood in case of transient bacteraemia (anachoresis) or from adjacently infected tooth (Narayanan and Vaishnavi 2010). The microbiology of pulpitis is variable and complex, in one study using a traditional culture method (Gajan *et al.* 2009), Gram positive bacteria were reported in 67.8 % of the cases of primary root canal infection. The two most prevalent genera among the collected samples were *Peptostreptococcus* and *Streptococcus* spp. Black-pigmented bacteria including *Prevotella* and *Porphyromonas* species were also seen in primary pulpal infections (Gajan *et al.* 2009). In general, species most frequently found in primary root canal infections usually belong to the genera *Streptococcus*, *Bacteroides*, *Fusobacterium*, *Prevotella*, *Porphyromonas*, *Treponema*, *Peptostreptococcus*, *Eubacterium* and *Actinomyces* (Sundqvist 1992; Siqueira *et al.* 2002). *E. faecalis* has been also frequently isolated from necrotic pulps indicating its possible pathogenic involvement in late stage pulpitis (Salah *et al.* 2008). Ayre and colleagues (Ayre *et al.* 2018) modelled a mixed species *ex-vivo* pulp infection using *S. anginosus* and *E. faecalis*. This study showed that although *S. anginosus* reached log phase more rapidly in the presence of *E. faecalis*, the mixed species

co-cultured with the tooth model did not show more extensive cell death, attachment or inflammatory response from the dental pulp than the single species culture. However, a higher inflammatory response was shown to be elicited by *E. faecalis* alone. This was proposed by the authors to be due to its higher tendency to attach to the pulp vasculature. This finding may support a role for *E. faecalis* in the pathogenesis of pulpitis. Using molecular checkerboard DNA-DNA hybridisation assay, of bacteria isolated from 53 infected teeth included in the study of Siqueira's team, Streptococci (mostly *S. anginosus*) were identified in 22.6 % of the samples followed by *Actinomyces* spp. in 9.4 %, and *E. faecalis* in 7.5 %. In asymptomatic lesions, the most prevalent species were *S. intermedius* (11.5 % of the cases), *E. faecalis* (11.5 %), and *S. anginosus* (7.7 %). *S. constellatus* was the most prevalent species in pus samples (25.9 % of the cases). Other species isolated frequently from abscessed teeth were *A. gerencseriae* (14.8 %), *S. gordonii* (11.1 %), *S. intermedius* (11.1 %), *A. israelii* (7.4 %), *S. anginosus* (7.4 %), and *S. sanguis* (7.4 %). Interestingly, *S. constellatus* was the only species positively associated with acute peri-radicular abscess (Siqueira *et al.* 2002).

A polymicrobial microbiota has been reported in cases of necrotic pulpal tissues (Siqueira *et al.* 2005) with species of Gram-negative anaerobes often isolated (Isabela N. Rôças *et al.* 2004). If the necrotic pulp remains *in situ* with no intervention, then the infection can proceed deeply into the periapical tissue causing apical periodontitis which may lead to the more serious condition of abscess or granuloma formation. The microbiology of apical periodontitis is usually complex and strict anaerobes are often predominant. It has been shown that the most prevalent taxa in the apical root canal system are *Olsenella uli*, *Prevotella baroniae*, *Porphyromonas endodontalis*, *Fusobacterium nucleatum*, *Tannerella forsythia* (Siqueira and Rôças 2009; Rôças *et al.* 2010). Species of streptococci were also identified in cases of apical periodontitis such as the viridans group *streptococci* and *S. anginosus* group (Rôças *et al.* 2010; Shweta and Prakash 2013). A systematic review correlating *E. faecalis* incidence in primary and persistence intraradicular infection has revealed that *E. faecalis* is more highly correlated with persistent intraradicular infections compared with untreated chronic periapical periodontitis (Zhang *et al.* 2015).

The commensal SAG has been recognised as an early coloniser involved in the initiation of pulpitis and in periapical abscesses. *E. faecalis* has also been found to correlate with persistence endodontic infections and to play a role in the pathogenesis of pulpitis. Therefore

it would be worthwhile to have a clear understanding of their prevalence and pathogenicity which would help treatment and/or prevention of such infections.

1.4.3 *Streptococcus anginosus* Group (SAG)

The *Streptococcus anginosus* group (previously known as the *S. milleri* group) is a subclass of viridans streptococci that comprises three separate streptococcal species: *S. anginosus*, *S. intermedius*, and *S. constellatus.* These bacteria are Gram positive, facultative anaerobes that exhibit variable haemolysis patterns (alpha, beta, or gamma) on sheep blood agar and enhanced growth when grown in the presence of CO_2. They are part of the normal microbiota of human oral and gastrointestinal tract but have a propensity for establishing abscesses and systemic infection. However, their precise mechanisms of virulence are not yet fully clarified, taking into account the differences in intrinsic pathogenic factors among the individual species of the group. It has been illustrated that SAG, particularly *S. intermedius* and *S. constellatus* have the ability to produce many enzymes such as hyaluronidase and chondroitin sulphate depolymerase (Homer *et al*. 1993; Jacobs *et al*. 1995). Bacterial-hyaluronidase catalyses host tissues hydrolysis of hyaluronic acid which in turn increases the permeability of the tissue enhancing the bacterial invasion. Amongst the viridans streptococci, *S. intermedius* has been shown to synthesize the broadest range of glycoprotein- and glycosoaminoglycan-degrading enzymes (Homer *et al.* 1993). In the context of pulpal infection, the production of these enzymes may contribute to the virulence and pathogenicity of SAG as the pulp tissue is rich in substrates for these enzymes. It has been found that 16 out of 45 clinical samples taken from patients with periodontal abscess were positive for SAG mainly *S. anginosus* (Fisher and Russell 1993). This has been supported by another study's finding which reported that, among dental plaque pathogens that were used as an inoculum for the formation of periapical abscess, SAG was the most frequently detected organism and the most prevalent in abscess lesions despite their low number in plaque (Okayama *et al.* 2005). These studies further highlight the importance of SAG in pulp infection especially if accompanied by other pyogenic bacteria such as *Prevotella intermedia* and *Porphyromonas endodontalis* where the likelihood to develop abscess and granuloma become significant (Trowbridge and Stevens 1992; Shinzato and Saito 1994; Young *et al.* 1996; Nagashima *et al.* 1999). Binding to fibronectin via the expression of cell surface adhesions by SAG members may contribute to their pathogenicity and some are able to bind to platelet-fibrin (Willcox

and Knox 1990; Kitada *et al.* 1997). A study has examined the adherence of SAGs to the matrix proteoglycans decorin and biglycan of soft gingival and alveolar bone. Of the SAGs members, *S. constellatus* showed the highest adherence followed by *S. intermedius* and the interaction is mediated by the conjugated glycosaminoglycan chain (Landrygan-Bakri *et al.* 2012). It has been shown that *S. constellatus* can resist phagocytosis in a murine model for pulmonary infections which has been proposed to be due to a polysaccharide capsule (Toyoda *et al.* 1995). Toxin production has been reported for SAG including the potential virulence factor intermedilysin, a cytolytic toxin of *S. intermedius* (Nagamune *et al.* 2000). Biofilm formation is considered to be relevant to virulence, and SAG biofilm formation potential has been studied and it has been documented that *S. constellatus* and *S. intermedius* have the capacity to form biofilm on abiotic materials which may contribute to their pathogenicity (Yamanaka *et al.* 2013).

1.4.4 *Enterococcus faecalis*

Enterococci are Gram positive facultative anaerobes with the ability to grow in the presence or absence of oxygen. Although enterococci are regarded as normal constituent of the human gastrointestinal microbiota, the bacteria have been associated with many infections of the urinary tract, surgical wounds, endocarditis and bacteraemia and chronic periapical periodontitis. They can survive in very harsh environmental conditions and can avoid antimicrobial challenges. Enterococcus species can grow at temperatures ranging from 10 °C to 45 °C, at extreme alkaline pH up to 9.6 and extreme salinity of 6.5 % NaCl. These may contribute to its ability to survive in the harsh environment of the root canal, especially filled ones, where there are nutritional deficiencies and limited means to escape the root canal medicaments. John et al. (2015) has described *E. faecalis* as the "endodontists' nightmare" as it has been identified in 24 % to 90 % of cases of failed root canals (Rôças *et al*. 2004; Sedgley *et al*. 2006; Stuart *et al*. 2006; Zoletti *et al*. 2006).

Many *E. faecalis* virulence factors have been identified and amongst the most extensively studied of them are aggregation substance, surface adhesin, sex pheromones, lipoteichoic acid, extracellular superoxide, gelatinase, hyaluronidase and cytolysin (hemolysin). Aggregation substance (AS), is involved in plasmid transfer and cell adherence and has been identified more frequently in pathogenic *E. faecalis* clinical isolates than isolates from healthy volunteers which emphasised the role of AS in enterococcal infection (Coque *et al.* 1995;

Elsner *et al.* 2000). Surface adhesins (SA) are also suggested to be involved in the pathogenicity of *E. faecalis*. *E. faecalis* surface protein (Esp) has been found to associate with the primary attachment and biofilm formation of *E. faecalis* on abiotic surfaces (Toledo-Arana *et al.* 2001). The gene ace has been suggested to involve in the binding of *E. faecalis* to extracellular matrix proteins (Nallapareddy *et al.* 2000). *E. faecalis* sex pheromone system has been studied and it has been proposed that resistance to antibiotics and other virulence traits, like cytolysin production can be passed in strains of *E. faecalis* by mean of sex pheromone system (Clewell and Weaver 1989). *E. faecalis* has been shown also to adhere to the root canal and dentinal walls forming a highly resistant biofilm (George *et al.* 2005). The bacteria adhere to the mineral part of the dentine probably through the effect of lipoteichoic acid (LTA) and to collagen via aggregation substances and surface adhesins which are likely to be expressed under stress (Rich *et al.* 1999) and are significant in initiating the enterococcal adherence to dentinal tubule walls (Hubble *et al.* 2003). Under conditions of nutrient limitations, *E. faecalis* can obtain nutrients from the dentine through the effect of bacterial hyaluronidase and other enzymes causing damage to the dentine tissue.

1.4.5 Pattern of microbial colonisation

To date, there have been two well characterised states of how bacteria can exist in nature and in the infected tissue; planktonic and biofilm form. In the root canal system, bacteria may be floating in the fluid phase of the main root canal unattached to any surfaces or can preferentially exist as attached aggregates to surfaces, forming matrix-enclosed biofilms (Siqueira *et al.* 2002). Any endodontic treatment strategy, to be successful, must take into account the pattern of bacterial existence in the root canal system. Planktonic bacterial cells are easier to be eradicated while sessile cells are more difficult and highly resistance to antimicrobial therapy.

1.4.5.1 Steps of biofilm formation

Biofilm establishment involves consecutive steps starting with initial reversible attachment to a biotic or abiotic surface (Sauer 2003). With the subsequent production of extracellular polymeric substance (EPS) which composes of DNA, proteins, lipids, and lipopolysaccharides of bacterial and host origin, bacterial cells irreversibly and firmly adhere to the surface (Okshevsky and Meyer 2015). The adsorbed cells then multiply, growing into microcolonies encapsulated in a layer of hydrogel. Finally, mature biofilm is settled with channels or pores

traversing the depth of it and free bacterial cells are released into the fluid phase maintaining the propagation of renewed communities and infection (Hall-Stoodley *et al.* 2004).

1.4.5.2 Adaptation mechanisms

One of the physiological and morphological surviving adaptation of pathogens is to develop into a protected community against environmental changes rather than a single individual. From this prospective, infected root canals may house microbial communities constituted of multiple populations (Mohammadi *et al.* 2014) which are mostly composed of different species (Socransky and Haffajee 2002; Socransky and Haffajee 2005). In such communities, microorganisms have to respond to different nutritional, chemical and oxygen gradients probably via new optimised phenotypic properties expressing a large set of genes which are proposed to be different from those expressed by the planktonic state of the particular species (Li *et al.* 2000). Emerging evidence has suggested that this gene expression might take place in a well-regulated manner (Davey and O'toole 2000; Sauer *et al*. 2002; Stoodley *et al*. 2002) and the so called two-component regulatory system is proposed to be involved which includes a histidine kinase and a response regulator (Hoch 2000; Miller and Bassler 2001; Scheie and Petersen 2004). To maintain cooperative ecological and functional balance of the biofilm realm, each population is suggested to be responsible for a functional niche (Siqueira and Rôças 2009). Bacterial cells are proposed to communicate with each other through the release, sensing and reaction to small diffusible signal particles; a mechanism known as quorum sensing, which is a basic chemical form of communication between different cells in the biofilm kingdom controlling a wide range of activities like nutrient requirement and pathogenicity (Harmsen *et al*. 2010; Li and Tian 2012).

The recent interest in such communication pathways has built a strong background for further investigation on how to develop anti-biofilm therapy via interfering, in some way, with these messaging strategies. For instance, an orthologue of *luxS*, required for the synthesis of autoinducer-2 signal (AI-2) which in turn is involved in interspecies communication, was identified and sequenced from *S. anginosus.* Inactivation of this *luxS* was found to result in isogenic mutant *S. anginosus* strain of diminished capacity to form biofilm (Petersen *et al.* 2006).

1.4.5.3 Mechanisms of antimicrobial resistance of biofilm

Biofilms may adopt antibiotic resistance mechanism/mechanisms to antagonise the effect of antibiotics. One of these proposed mechanisms is through encouraging the growth of certain species beneficial to the biofilm realm that are already resistant to the particular antibiotic perchance via quorum sensing communication and gene transfer (Svensäter and Bergenholtz 2004). The association between *LuxS* gene, which has been shown to be involved in biofilm formation, and antibiotic susceptibility was studied (Abdul-Majeed 2008, PhD thesis). It was concluded from the study that *S. anginosus luxS* mutant displayed diminished ability to endure several subinhibitory concentrations of ampicillin and erythromycin compared to wild type strain.

The so called 'persister cells', slow or non-growing dormant cells, is another offered biofilm antibiotic-resistant mechanism as the slow growth rates render the persister cells less sensitive to antibiotic treatment therefore they can survive and act as reservoirs providing cells that regrow causing relapsing chronic infection (Mah 2012)

The availability of nutrients also affects the biofilm's susceptibility to antibiotics. This has been supported by the finding that nutrient-limited biofilm of *P. aeruginosa* became highly antibiotic tolerant (Nguyen *et al.* 2011). Some chemical changes that occur in the biofilm such as oxygen depletion and acidic wastes accumulation have been reported to antagonise the effect of antibiotics (Athanassiadis *et al.* 2007).

1.5 Current treatment options of dental pulpitis

Worldwide, the age-standardised prevalence of untreated dentine carious lesions in the primary dentition in the global population has remained static over two decades (1990-2010) at about 9 % while the prevalence of untreated caries in the permanent dentition was 35 %. This means caries continued to be the most prevalent health condition across the globe in 2010, affecting around 2.4 billion people (Kassebaum *et al.* 2015). Uncontrolled caries leads to pulpitis which is considered as one of the most frequent causes for patients seeking emergency dental care (Keenan *et al.* 2005). Clinically, patients with pulpitis are presented to the clinic with increased sensitivity in the tooth, discomfort or pain. Treatment depends on the severity of the condition, which has two clinical classifications: reversible or irreversible pulpitis. Many factors play a role in the diagnosis of pulpitis including history and mode of the

pain, sensitivity of the affected tooth to different stimuli such hot and cold, tapping (percussion test) and electrical current pulp test (Mejàre *et al.* 2012). However, asymptomatic pulpitis can also occur especially in the elderly (Farac *et al.* 2012). In irreversible pulpitis, the damage is more severe and management must include removal of all the necrotic pulp in order to prevent further spread of infection into the periapical tissue. For reversibly infected pulp, vital pulp therapy can be considered.

1.5.1 Vital pulp therapy (VPT)

Vital pulp therapy is a procedure that aims to preserve and maintain pulp tissue that has been affected but not completely destroyed by deep caries, trauma or fault restorative procedures. The tissue must be vital and not necrotic because VPT is based on the regenerative potential of the dentine-pulp complex to promote pulp tissue healing and facilitate the formation of the reparative dentine (Aguilar and Linsuwanont 2011). It does involve the removal of the offending irritants and placement of a protective material either directly or indirectly over the exposed pulp (Hargreaves and Berman 2015). Importantly, a tight-sealed restoration must be overlaid on to decrease bacterial leakage from the restoration-dentine interface. The ultimate goal of this technique is to promote root development and apical closure in addition to the protective resistance to mastication forces compared with a root-canal-filled tooth (Ward 2002; Bergenholtz and Spångberg 2004; Dammaschke *et al.* 2010; Aguilar and Linsuwanont 2011; Cohenca *et al.* 2013). Survival rates of teeth treated endodontically have been reported to be lower than those of as vital teeth particularly in molars therefore, every effort should be done to preserve a vital tooth (Caplan *et al.* 2005). The decision about whether to undertake VPT to an affected pulp is difficult. This depends on the clinical status of the pulp tissue which is in turn difficult to predict. The traditional school of thought is that VPT should only be performed in teeth with signs and symptoms of reversible pulpitis (Al-Hiyasat *et al.* 2006). However, it is not easy nor precise to predict the severity of the inflammation of the pulp by clinical assessment based on sign and symptoms presented by the patients or through electric, thermal or radiographic assessment. Clinical diagnosis have been evidenced to contradict histology figures on occasions (Mejàre *et al.* 2012). Moreover, successful treatment outcome has been reported in vital teeth with signs and symptoms of irreversible pulpitis and periapical lesions (Mejare and Cvek 1993; Matsuo *et al.* 1996; Teixeira *et al.* 2001; Asgary *et al.* 2015). It has been recommended that VPT should be performed only in young

patients because of the high healing capacity of pulp tissue (Cvek 1978; Ward 2002). However preservation of the pulpally involved permanent tooth should also be considered in older people (Aguilar and Linsuwanont 2011). Therefore, it would be worth considering VPT before embarking on the more invasive and destructive root canal therapy.

The differing methodologies followed in the different studies might give rise to difficulties in comparing treatment outcomes. However, most studies have noticed a favourable treatment outcome, which seems to be inconsistent with the clinical viewpoint that the VPT outcome is uncertain (Dammaschke *et al.* 2010). Many factors should be considered to achieve the best therapeutic result of VPT including an accurate diagnosis of the pulp status, skilful cleaning of the carious lesion, appropriate sealing to prevent microbial leakage in addition to the use of aseptic technique during the procedure (Stanley 1989; Nair *et al.* 2009; Ali *et al.* 2012).

1.5.1.1 Indirect pulp capping

Indirect pulp capping (IPC) is the procedure that can be performed as an attempt to preserve a carious dentine to avoid pulp exposure (American Academy of Pediatric Dentistry 2016). A high survival rate (> 90 %) has been reported for permanent teeth treated with IPC with clinical and radiographic signs of healing (Tziafas 2004; Maltz *et al.* 2007). The bioactive lining material should be able to stimulate the odontoblast cells to form reactionary and reparative dentine thus promote remineralisation of the exiting dentine and therefore encouraging the dentine-pulp complex (Hilton 2009).

IPC can be performed by either of two approaches: the *incomplete caries removal with no re-entry* -where the residual caries is sealed into the cavity with no further intervention- and the *stepwise* or *two-step excavation* approach-where the caries is removed over two stages with a gap of few months in between to allow for the formation of the reparative dentine before complete caries removal. Although the evidence is not satisfactory, it has been shown that there is no difference in the outcome of either the complete or incomplete caries removal (Ricketts *et al.* 2009). However, other systematic review concluded a better outcome with incomplete caries removal in comparison to the stepwise caries removal (Thompson *et al.* 2008; Schwendicke *et al.* 2013). This provides evidence that the restoration can tight-seal the lesion from the oral environment effectively and subsequently depriving bacteria of nutrients.

1.5.1.2 Direct pulp capping

Direct pulp capping (DPC) is defined as the treatment of vital pulp exposed due to mechanical or traumatic insult by sealing the pulpal wound with a biomaterial placed directly on exposed pulp. This material is supposed to facilitate formation of reparative dentine and maintain and protect a vital pulp (American Association of Endodontists guideline, 2003). The progenitor /stem cells in the underlying uninfected vital pulp is stimulated by the initiated inflammatory response from the damaged odontoblasts to produce reparative dentine (Tziafas 2004). Growth factors such as TGF family of cytokines released from the dentine matrix can induce stem cells differentiation. A lower success rate is seen to be associated with carious-associated exposed dentine indicating a possible important role of the microorganisms in the outcome of DPC (Barthel *et al.* 2000; Al-Hiyasat *et al.* 2006; Dammaschke *et al.* 2010). It is therefore recommended that a microbial-tight restoration should be placed immediately on the exposed cavity besides following other aseptic techniques.

A clinical study of DPC performed on 44 teeth with carious-exposed pulp demonstrated a success rate of 81.8 %. The study declared no association between patients' age, sex, presence of spontaneous pain and size of exposure on the outcome. However, the extent of bleeding was recorded as an influential factor on the pulp tissue healing (Matsuo *et al.* 1996). Although still not clinically approved, Miniature pulpotomy procedure (MPP) has been proposed by Asgary and Ahmadyar (Asgary and Ahmadyar 2012) to improve the treatment outcome of DPT. They claimed that removal of infected dentine chips/damaged pulp tissue ,specially injured odontoblast cells, would improve proximity/interaction of pulp covering agents to undifferentiated mesenchymal/stem cells and offer better control of bleeding and creating an improved seal using pulp covering agents.

1.5.1.3 Pulpotomy

Pulpotomy can be partial or full, in either case the inflamed tissue beneath pulp exposure is removed and a bioactive material is placed over the remaining pulp to allow for healing and reparative dentine formation (American Association of Endodontists guideline, 2003). In partial or Cvek pulpotomy, the inflamed tissue is removed to the level of healthy coronal pulp tissue. In full or complete pulpotomy, a surgical removal of the entire coronal portion of the vital pulp is performed to preserve the vitality of the remaining radicular portion. This procedure is followed when there is inflammation of the coronal part of the pulp. The

homeostasis should be achieved and a biomaterial should be placed on the remaining pulp tissue. A success rate of 93-96 % has been reported for partial pulpotomy (Nosrat and Nosrat 2002; Kiatwateeratana *et al.* 2009). A recent study compared IPC and pulpotomy of 80 primary molars, follow-up evaluation revealed 100% clinical success in teeth treated with mineral trioxide aggregate (MTA) pulpotomy and IPC. One radiographic failure of internal resorption detected at 3 months with MTA pulpotomy which was not statistically significant. This study findings may redirect the vital pulp therapy modalities towards IPC which offers several advantages over pulpotomy like less potential side effects, non-invasive, reduced chair time, child cooperation and cost-effectiveness. However, the cases were followed for 3 months which is not enough to make a conclusive statement (Vidya *et al.* 2015). A high pulpotomy success rate between 96-100 % was recently reported on ferric sulphate and biodentine-treated primary molars respectively followed for 9 months (Sirohi *et al.* 2017). It has been shown that success rate is not influenced by the age and maturation of the root apex nor the restoration material used in pulpotomy (Alqaderi *et al.* 2016). In case of necrotic pulp, root canal therapy must be initiated. The whole affected pulp should be removed, the root canal system disinfected, the canals filled with inert fillers and then sealed properly to prevent microleakage.

1.5.2 Agents used in vital pulp therapy

The success of the pulp capping procedure greatly depends upon the circumstances under which it is performed and the prognosis depends upon the age, type, site and size of pulp exposure. In addition to this the pulp capping material should have the following ideal properties like: (Qureshi *et al.* 2014)

- Stimulate reparative dentine formation
- Maintain pulpal vitality
- Release fluoride to prevent secondary caries
- Bactericidal or bacteriostatic
- Adhere to dentine
- Adhere to restorative material
- Resist forces during restoration placement and during the life of restoration
- Sterile

- Radiopaque
- Provide bacterial seal

1.5.2.1 Calcium hydroxide

Calcium hydroxide is considered as a major player in the development of VPT and has been considered as the gold standard for direct pulp capping materials for decades (Dammaschke *et al.* 2010). It can irritate pulp tissue cells thus stimulating pulp defense and repair (Cox *et al.* 1996). It also shows the potential to extract growth factors from the dentine matrix which helps in dentine regeneration (Graham *et al.* 2006). The alkaline nature of this compound (> pH 12) has antimicrobial activity which add a benefit in treating carious lesions (Cox *et al.* 1996; Barthel *et al.* 2003). Despite the long history, variable long term outcomes have been reported with the use of calcium hydroxide (Barthel *et al.* 2000; Robertson *et al.* 2001; Auschill *et al.* 2003). In a review of 14 clinical studies, including over 2,300 cases of calcium hydroxide pulp capping, a success rate of > 90 % was reported when pulp therapy performed by experienced clinicians (Dammaschke *et al.* 2010). However, a lower success rate was also reported. For instance, in one study, DPC with calcium hydroxide revealed a success rate of 61 % (219 teeth out of 359 teeth) with a trend of decreasing success by age (Auschill *et al.* 2003). Success rate of calcium hydroxide in primary molars was 79.9 % for teeth restored with a stainless steel crown and 60 % for those restored with amalgam followed for 1 year (Sonmez and Duruturk 2010). The low success rates reported might be due to the disadvantages held by the material. The high solubility of the self-cure formulation makes the pulp capping material subjective to dissolution over time (Prosser *et al.* 1982). However, a dentine bridging has been found to be initiated by the time calcium hydroxide dissolute (de Lourdes Rodrigues Accorinte *et al.* 2006). Moreover, calcium hydroxide lacks adhesive qualities and therefore provides a poor seal (Ferracane 2001). The presence of so-called “tunnel defects” in reparative dentine formed underneath calcium hydroxide pulp caps is an added criticism to the use of this material (Cox *et al.* 1995; Kitasako *et al.* 2008), though this happens only occasionally (de Lourdes Rodrigues Accorinte *et al.* 2006)(Silva *et al.* 2006). The formed tunnels and pores can provide a pathway for microbial leakage.

1.5.2.2 Mineral Trioxide Aggregate (MTA)

Mineral Trioxide Aggregate has gained considerable attention as a direct pulp capping agent in recent years. It is basically composed of calcium oxide in the form of tricalcium silicate, dicalcium silicate and tricalcium aluminate. Bismuth oxide is added for radiopacity (Camilleri 2008). Upon MTA reaction with water, calcium hydroxide is produced (Camilleri and Pitt Ford 2006; Camilleri 2008) which gives rise to the MTA's biocompatibility (Camilleri *et al.* 2005). Therefore MTA shares most of its properties with calcium hydroxides except that that MTA provides some seal to tooth structure (Ferk Luketić *et al.* 2008). As with calcium hydroxide, MTA has some disadvantages like high solubility, demonstrating 24 % loss after 78 days of storage in water (Fridland and Rosado 2003). Although it is aesthetically acceptable, the presence of iron in the MTA formulation may darken the tooth (Aeinehchi *et al.* 2003). A potential drawback for the use of MTA is its prolonged setting time (between 2 - 45 min) (Torabinejad *et al.* 1995; Islam *et al.* 2006) which requires the capping to be done in a two-step procedure or using a quick-setting liner to protect the MTA. MTA is also difficult to handle and offers low cost-effectiveness when compared to calcium hydroxide.

As a direct pulp capping material in carious-exposed pulp of primary teeth, MTA was found to be as successful as calcium hydroxide in cases followed for 24 months (Tuna and Ölmez 2008). A similar finding was obtained in partial pulpotomy of permanent molars with deep caries where MTA showed clinical success rate comparable to calcium hydroxide with an average of 34.8 ± 4.4 months (Qudeimat *et al.* 2007). In mechanically exposed pulp, an equal effectiveness of MTA and calcium hydroxide was reported for 2-months followed permanent premolars (Accorinte *et al.* 2008). While another two studies conducted on mechanically exposed pulp revealed a superiority of MTA over calcium hydroxide (Chacko and Kurikose 2006; Nair *et al.* 2009). A recent systematic review and meta-analysis aimed to compare the effect of MTA and calcium hydroxide for direct pulp capping suggested that MTA was superior to calcium hydroxide in direct pulp capping resulting in a lower failure rate (Zhu *et al.* 2015).

1.5.2.3 Adhesive resins

Self-etching adhesive systems have been provided as pulp capping materials. Reported outcomes were poor including the induction of unresolved inflammatory reaction and low grade pulp repair (Prager 1994; Accorinte *et al*. 2008; Cui *et al*. 2009). This might be due to

their vasorelaxant effect (Maddux *et al*. 2002) promoting bleeding which in turn may compromise the adhesive polymerisation and result in an increase in their cytotoxicity. It has therefore been suggested that the adhesive resins are unacceptable as pulp capping agents (Ghoddusi *et al*. 2014).

1.6 Future advances in the materials and biological sciences

The use of biomaterials has been extended recently to offer a therapeutic application in endodontics. Growth factors such as bone morphogenic proteins and TGF-beta have been reported to be involved in the mechanism of reparative dentine formation (Tziafas *et al*. 2000). Recombinant human BMP-2, -4, and -7 are currently commercially available for experimental use and clinical trials. Other biomaterials such as bioceramic, biodentine, Emdogain (STRAUMANN, USA), propolis, tricalcium phosphate cement, and some other bioactive materials and even stem cells have also been suggested for VPT (Nakamura *et al.* 2002; Min *et al.* 2009; Guven *et al.* 2011; Hirschman *et al.* 2012; Nowicka *et al.* 2013). They have shown to induce pulp tissue to produce tertiary dentine either through direct or indirect contact. The effect on dentine thickness has been shown to be dose-dependent (Ranly and Garcia-Godoy 2000). Despite the apparent promising results that can be obtained, further evaluation of the use of these materials as new strategies for VPT is required.

Against all these data, it seems that VPT still needs further development of agents that combine properties of efficient dressing with growth factors in an easily handled carrier with advanced antimicrobial and anti-inflammatory properties delivered to the clinic with reasonable price (Ranly and Garcia-Godoy 2000; Fuks 2008). Bacterial biosurfactants, preferably of probiotic origins, can be one option.

1.7 Probiotics

As living microorganisms, probiotics are capable of producing many by-products such as organic acids, bacteriocin and biosurfactants, with broad different activities such as antimicrobial, antiadhesive and immune-modulatory effects and these have started to be used in industry and medicine.

1.7.1 What are probiotics?

As a word, probiotic is derived from Latin word “pro”-for and Greek word “biotic”- life. The international definition of probiotics, which has been approved by the United Nations Food and Agriculture Organization (FAO) and the World Health Organization (WHO), is that “probiotics are live microorganisms which when administered in sufficient quantity confer a health benefit on the host”. The key points of this definition are that the microbes must be alive when introduced to the host by any means, and they must have evaluated health advantages in the host (Hamilton-Miller *et al.* 2003). Before their existence by modern science, the use of live microorganisms in food has a long history.

The first observation of using bacteria for health benefits was documented by Eli Metchnikoff, the Nobel Prize winner for his pioneering discovery in the field of probiotics (McCann 2004). He had observed that Bulgarian peasants consuming diet high in fermented milk had a reduced incidence of enteric illnesses and lived longer. Their fermented food and intestines were found to contain bacterial strains including *Lactobacillus bulgaricus.* This successful treatment resulted in the development of different health resorts in Europe in the early 1900s. Lilley and Stillwell in 1965 were the first who proposed the term “probiotic” as opposed to “antibiotic”, while *Lactobacillus acidophilus* was the first probiotic species introduced by Hull et al. in 1984 followed by *Bifidobacterium bifidum* by Holcombh et al. in 1991 (cited in Gupta 2011).

Probiotics can be subcategorised as probiotic drugs which are proposed to treat or prevent disease, probiotic foods including foods, food ingredients and dietary supplements, direct-fed microorganisms (probiotics for animal use) and genetically modified ones which are the designer probiotics (Sanders 2009). The majority of probiotics derive from the *Lactobacillus* or *Bifidobacterium* genera, followed by *Saccharomyces Boulardii* (a yeast) and less commonly used are strains of *Escherichia coli* or *Bacillus coagulans.* Virus is the category of microbe which is typically not regarded to be a probiotics and using them as live vaccines is considered to be outside the realm of probiotics (Sanders 2009). In 1994, as commonly prescribed antibiotics were becoming of less value because of bacterial resistance, the WHO considered probiotics to be the next most important immune defence mechanism (Gupta 2011). This new thought has fed research for studying the possible application of probiotics in medicine and dentistry.

1.7.2 Criteria for selecting probiotics

In 2002, a joint report by the FAO and WHO outlined a number of certain selection criteria and intrinsic physio-chemical properties for bacteria to be considered as probiotics (WHO 2002). The minimum requirements needed for probiotic status include:

1. The assessment of strain identity (genus, species, strain level); from the current available evidence, it has been suggested that probiotics effects are strain specific. It is important to link a strain to a specific health effect as well as to enable surveillance and epidemiological studies.
2. Screen potential probiotic strains *in vitro*: it is recommended that *in vitro* tests should be done before any subsequent animal or human *in-vivo* assessment. These *in vitro* tests performed on probiotics include: resistance to gastric acidity, bile acid and digestive enzymes, antimicrobial activity against potentially pathogenic bacteria, adherence ability to mucus and/or human epithelial cells, bile salt hydrolase activity, surface anti-adherence activity against pathogens and resistance to spermicides (applicable to probiotics for vaginal use).
3. Safety assessment: requirements for proof that a probiotic strain is safe and without contamination in its delivery form. As a group, probiotics are generally regarded as safe (GRAS) organisms. However, four kinds of side effects are theoretically probiotics may be responsible for (Marteau 2001) which are systemic infections, harmful metabolic effects, undue immune stimulation in prone individuals and gene transfer.

   The required safety assessment includes the determination of antibiotic resistance profiles, estimation of certain metabolic activities such as D-lactate formation and bile salt deconjugation which is unwanted action in the small intestine, determination of side effects in human trial, post market epidemiological surveillance of adverse effects in consumers, haemolytic activity and toxin production. It is recommended that animal trials must be undertaken before starting human ones. Very few studies reported the possibility of systemic probiotic infections (Oggioni *et al*. 1998, Mackay *et al*. 1999, Rautio *et al*. 1999). However, in these rare examples all patients had serious underlying medical conditions.

4. *In-vivo* studies for substantiation of health effects in the target host. The efficacy outcome of studies on probiotics should be proven beneficial in human trial such as statistical and biological significant improvement in disorder, signs and symptoms, quality of life; reduction in incidence and prevalence of disease or rapid onset of relief. Physiological and technological strength are required for potential probiotic strains, and many of the clinically promising probiotics are unfortunately rendered unused because they are physiologically and technologically fragile (Mattila-Sandholm *et al.* 2002)

1.7.3 Mechanism of probiotics action

The mechanisms underlying the beneficial effects of probiotics are largely unknown but are likely to be multifactorial (Figure 1.2). Different probiotic strains may use different mechanisms for their action and some might have multiple activities and can influence different stages that are considered their action targets (Sherman *et al.* 2010). The proposed mechanisms of probiotic action could be parallel to those assumed for the gut as below (Bermudez-Brito *et al.* 2012):

1.7.3.1 Enhancement of the epithelial barrier

Intestinal barrier is considered a major defence mechanism used for maintaining epithelial integrity. When this barrier function no longer exists, foreign antigens from bacterial or dietary origins can gain access to the submucosal layers and induce inflammatory reactions which may cause intestinal disorders, for example inflammatory bowel disease (Hooper *et al*. 2001; Hooper *et al*. 2003; Sartor 2006). It has been found that the consumption of non-pathogenic bacteria such as probiotics might contribute to intestinal barrier function but the precise mechanism by which probiotics boost intestinal barrier function is not precisely known.

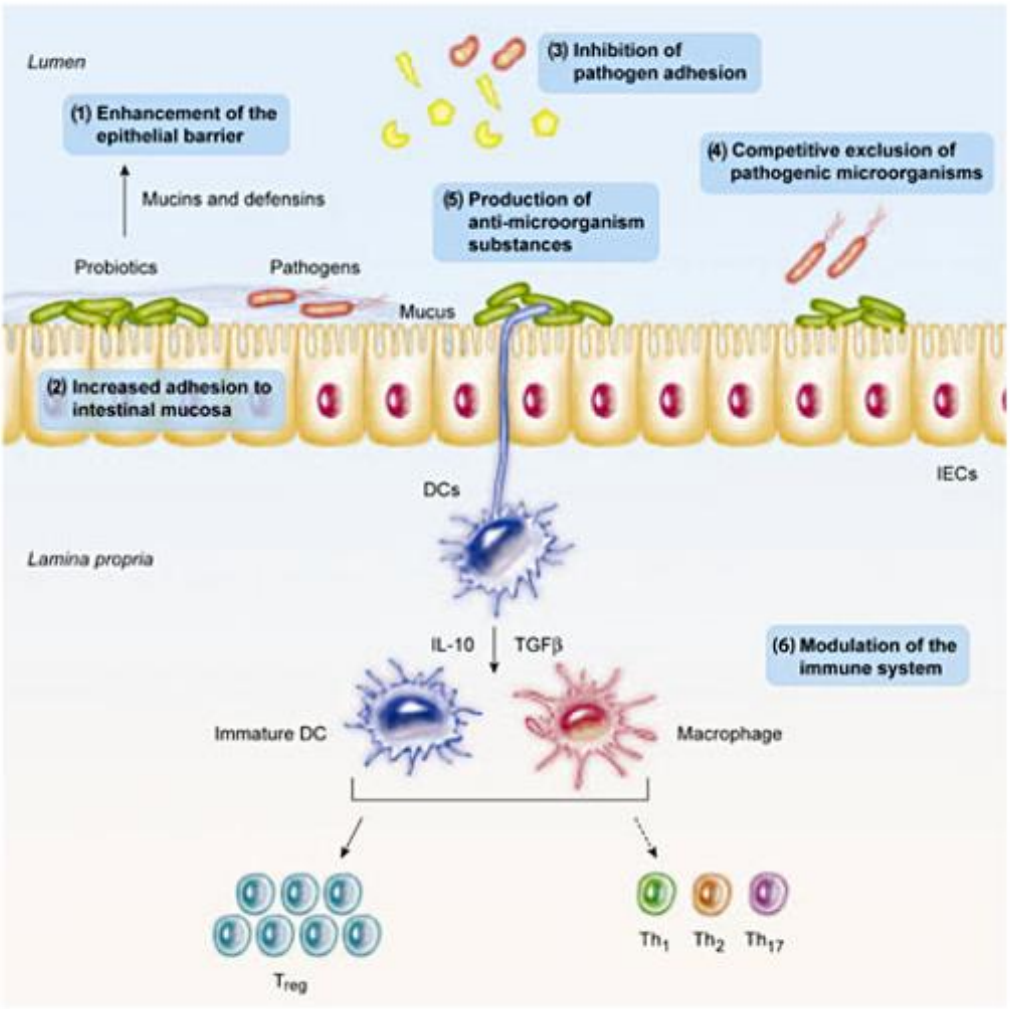


Figure 1.2 **Major proposed mechanisms of action of probiotics**. Cited from (Bermudez-Brito *et al*. 2012).

Anderson et al. (2010) found that one possible probiotics mechanism was the enhanced expression of genes involved in tight junction signalling. In another study conducted to investigate the influence of certain probiotic species in T84 cell epithelial barrier model (Hummel *et al.* 2012), these probiotic strains were found to modulate many genes coding adherence junction proteins beside enhancing the phosphorylation of adherence junction proteins therefore positively controlling these barrier functionality.

It was proposed that using probiotics may contribute to the strengthening of the epithelial

barrier by inhibition of cytokine-induced epithelial destruction which is a feature of inflammatory bowel disease. In two pieces of research conducted by Yan and his colleagues (Yan and Polk 2002; Yan et al. 2007) on *Lactobacillus rhamnosus* GG (LGG), two novel peptides known as p40 and p75 secreted by LGG were isolated and purified and shown to supress cytokine-induced cell apoptosis in two different intestinal epithelial cell models. Each of these purified protein preparations activated the anti-apoptotic Akt/protein kinase B, inhibited cytokine-induced epithelial cell apoptosis, and promoted cell growth in human and mouse colon epithelial cells and cultured mouse colon explants. TNF-induced colon epithelial damage was significantly reduced by p75 and p40. Immunodepletion of p75 and p40 from LGG-CM reversed LGG-CM activation of Akt and its inhibitory effects on cytokine-induced apoptosis and loss of intestinal epithelial cells. In addition, another study carried out by Tao and his co-researchers revealed that LGG secreted low molecular weight peptides which induce the expression of cytoprotective heat shock protein in gut epithelial and this may contribute to the beneficial effect of LGG as probiotic (Tao *et al.* 2006).

Enhancing mucus secretion could be a proposed mechanism by which probiotics exert their healthy intestinal effect. Many studies have investigated this property but only *in vitro*. Further studies are required to test the *in-vivo* adherence of probiotics which is a key determinant of altering mucin expression in human intestine (Mattar et al. 2002; Mack et al. 2003). *Lactobacillus acidophilus* was shown to increase *MUC2* expression in HT29 cells independent of attachment (Kim *et al.* 2008). Since only few studies have been conducted *in vivo*, the results are inconsistent and further *in vivo* investigation are really needed to culminate into a conclusive statement. For instance, in three different studies, it was reported that VSL3, which contains some *Lactobacillus* species, improved the expression of MUC2 , MUC3 and MUC5AC in HT29 cells (Otte and Podolsky 2004), while the same product did not demonstrate any effects on mucin expression when given to mice for 14 days (Gaudier *et al.* 2005). Conversely but interestingly, VSL3 causes a 60-fold increase in MUC2 expression and a growth of mucin secretion when rats were given the same dose for 7 days (Caballero-Franco *et al.* 2007).

1.7.3.2 Increased adhesion to surfaces and anti-adhesive effects

Adhesion (at least transient) to oral/intestinal mucosa is one of the required elements for colonisation and is a key for the interaction between the probiotics and the host tissues. Good

adhesion places bacteria and host surfaces (fluids or epithelial tissues) in close contact thus assisting more probiotic effects such as immune modulation and pathogens antagonism (Juntunen et al. 2001; Perdigon et al. 2002; Hirano et al. 2003). It has been reported that several *Lactobacillus* proteins promoted adhesion to mucous (Van Tassell and Miller 2011). For example, surface adhesin displayed by *L. acidophilus* is assumed to mediate this attachment *in vitro* (Buck *et al.* 2005). Proteins are mainly involved in adhesion, however saccharide moieties and lipoteichoic acids have also been associated (Vélez *et al.* 2007).

The probiotic adhesiveness to epithelial cells and the subsequent blocking adherence of pathogens is considered as one mechanism of the probiotic to exert their effects on the host. This antiadhesive effect could be the result of competitive binding to the same receptors or due to enhanced mucin production. It has been found that co-culturing of inHT20-MTX cells with *L. plantarum* 299v or *L. rhamnosus* GG actually enhanced induction of MUC3 which in turn inhibited the adhesion of enteropathogenic *E. coli* strain E2348/69 (Mack *et al.* 2003). Other mechanisms of antiadhesiveness could be degradation of carbohydrate receptors by secreted proteins, formation of established biofilm, production of receptors analogue or biosurfactant production (Oelschlaeger 2010).

Orally, the colonisation ability of 17 different probiotic strains has been tested *(Haukioja et al*. 2006). Lactobacilli demonstrated better adherence to saliva-coated surface, thus they may compete for the same binding sites with *Fusobacterium nucleatum* which explains its reduced colonisation property. Accordingly, probiotics may have an effect on biofilm formation and on modification of the oral microflora. Moreover, probiotic strains can affect the structure of salivary pellicle on hydroxyapatite and by that means inhibit *Streptococcus mutans* adherence *in vitro* (Haukioja *et al*. 2006). A significant weakness in these *in-vitro* studies is their restricted ability to reintroduce consistent and reliable environmental conditions. *In-vivo* studies, using simulated biofilm formation to assess adhesion, can provide more reliable results that might explain the phenomena taking place.

1.7.3.3 Competitive exclusion of pathogenic microorganisms

The means by which one microorganism eliminates or lowers the growth of another one are variable and could be due to establishment of hostile microenvironment, abolition of existence bacterial receptor sites, formation and secretion of antimicrobial products and other selective metabolites and competitive exhaustion of essential nutrients (Rolfe 1991).

Specific probiotic adhesion to intestinal mucosa may inhibit pathogenic strains from colonising the gut (Servin 2004). The competitive effect of probiotics has been studied using human mucosal cells as well as in animal models. It was observed that the potent adhesiveness of *L. rhamnosus* to a colon epithelial cell line put forward the possibility that an avid interaction between the probiotic bacterium and the host tissue might be modifying intra-cellular processes and subsequently inhibiting the internalisation of pathogenic enterohemorrhagic *E. coli* (Hirano *et al.* 2003). Bacteria can change the surrounding environment to be hostile for their opponents such as the formation and secretion of lactic and acetic acid (Schiffrin and Blum 2002). Another more important example is competition for iron which is essential for all bacteria apart from lactobacilli which do not require it for their natural growth and survival (Weinberg 1997). *L. acidophilus* and *L. delbrueckii* are able to bind ferric hydroxide at their cell surface, rendering it unavailable to pathogenic microorganisms (Elli *et al.* 2000). Yet, the *E. coli* strain, Nissle (EcN) is dependent on iron and able to efficiently compete with the pathogenic strains for this limited supply because it encodes at least seven iron uptake systems (Grozdanov *et al.* 2004; Große *et al*. 2006).

1.7.3.4 Production of antimicrobial substances

Production of substances with antimicrobial activity is one of the proposed mechanisms of probiotic actions. These antimicrobials include organic acids, hydrogen peroxide, deconjugated bile acids and inhibitory substance like bacteriocins and biosurfactants. Organic acids, mainly lactic and acetic acids, have potent antibacterial activity against Gram-positive bacteria (Alakomi *et al*. 2000; De Keersmaecker *et al*. 2006; Makras *et al*. 2006). The cidal power of these organic acids involves the reduction of intracellular pH and dissipation of the membrane potential (Kashket 1987; Ljungh and Wadström 2009). Lactic acid bacteria isolated from human oral cavity were observed to have antimicrobial activity against *Streptococcus mutans* and *Porphyromonas gingivalis* at highly acidic pH (Sookkhee *et al.* 2001). Hydrogen peroxide is considered as a non-specific antimicrobial especially against urogenital infection and bacterial vaginitis (Reid 2008; Pithva *et al.* 2011). Its antibacterial activity is attributed to its strong oxidizing effect on the bacterial cell and to the destruction of basic molecular structures of cell proteins (Lindgren and Dobrogosz 1990).

Bacteriocins are antimicrobial proteinaceous compounds produced by some probiotics and can have narrow or broad spectrum activity (Rogelj and Matijašić 1994; Cotter *et al*. 2005). These antimicrobial compounds have received considerable interest owing to their significant prospective usage in the food industry as natural preservatives (biopreservatives). The family of bacteriocins embraces different peptides and proteins of different sizes, microbial targets and mechanism of action and immunity (Garneau *et al.* 2002). Several mechanisms have been proposed for different bacteriocins effects. However, it was found that the majority of bacteriocins destroy susceptible bacteria by membrane permeabilization or by interference with essential enzymes; for instance, Nisin has been shown to form a complex with the cell wall precursor lipid II, in that way hindering cell wall biosynthesis. Afterward the complex aggregates and integrates with additional peptides to form a pore in the bacterial membrane (Pithva *et al.* 2011). Orally, bacteriocins have been proposed as one of different collective ways of preventing dental caries (Balakrishnan *et al.* 2000) via competition for the same target receptors and subsequent pathogen-attachment inhibition. An inventive therapeutic approach should be considered on the basis of the antimicrobial molecules produced by probiotics, which could be utilised as a potential foundation of new, antibiotic-like molecules. Biosurfactants derived from probiotics have also been shown to exert potential antimicrobial activity against a number of pathogenic bacteria and this will be reviewed later on this chapter.

1.7.3.5 Antitoxin effects

Bacterial toxins can be considered as key virulence factors. The antidiarrheal activity of probiotics can be attributed to their ability to protect the host against toxins. Two strains of *Bifidobacterium* were demonstrated to inhibit Shiga toxin expression of *E. coli* (STEC) O157:H7 *in vitro* as well as in mice model. High concentration of acetic acid production was thought responsible for inhibition of Shiga toxin expression (Asahara *et al.* 2004).

1.7.3.6 Modulation of the immune system

Probiotics, live or dead, or their-derived components can influence host immune system in one or another way. Either by direct adhesion of probiotics to host cells or via released-soluble compounds, a signalling cascade could be triggered leading to immune modulation. Grangette et al. (2005) revealed probiotic activity of *L. plantarum* when a mutant strain with

enhanced anti-inflammatory effect was shown to dramatically reduce pro-inflammatory cytokine secretion by peripheral mononuclear and monocyte cells of murine colitis model which was TLR-2 independent. It has been shown that gut mucosal barrier integrity can also be protected by probiotic against the damaging effects of enteropathogenic *E. coli* in a TLR-independent way (Zyrek *et al.* 2007). This study provided evidence that *E. coli* Nissle 1917 (EcN) can induce a principal signaling effect leading to the renewal of a disrupted epithelial barrier. This is conveyed via silencing of PKCzeta and the redistribution of ZO-2. The authors suggested that these properties may contribute to the reported efficacy in the treatment of inflammatory bowel diseases and in part justify the probiotic nature of EcN. Moreover *E. coli* Nissle 1917 (EcN) has been suggested to secrete a factor which suppresses the TNFα-induced IL-8 transactivation through mechanisms different from NF-kappaB inhibition when cultured with human colonic epithelial cell line HCT15 (Kamada *et al.* 2008).

While genomic DNA of pathogenic bacteria induces systemic inflammatory effects, probiotics genomic DNA has been shown to have systemic anti-inflammatory activity (Rachmilewitz *et al.* 2004). The reason for such differences is unknown. Anti-inflammatory probiotic effects can encompass the induction of Treg cells as well. It has been observed that subcutaneously administered probiotics exerted anti-inflammatory effects not only in colitis model but in arthritis treatment as well (Sheil *et al.* 2004). An *in vitro* study which was carried out by Takeda et al. (2006) revealed that *L. casei* Shirota induced IL-12 production which subsequently causes an increase in natural killer (NK) cells activity. Although these are examples of strain-specific activity, the molecular basis and clinical significance are required to be extensively investigated especially *in vivo*.

1.7.4 Colonisation and safety of probiotics in the oral cavity

Despite their positive intestinal activities, the effects of most intestinal probiotics on the oral tissue and microbiota remain insufficiently identified. Some of them are known to have positive effects on the oral health. Many *in-vitro* and *in-vivo* studies have indicated that the probiotics ability to colonise the oral cavity is variable and dependent on the probiotic strains, products, and also host (Busscher, Mulder, *et al.* 1999; Krasse *et al.* 2005; Haukioja, Yli-Knuuttila, *et al.* 2006; Yli-Knuuttila *et al.* 2006; Maukonen *et al.* 2008).

Some probiotic species such as *Lactobacillus* and *Streptococcus* appear to be able to inhabit the oral cavity of certain people so long as the products containing them are regularly used. It has been found that *S. salivarius* K12 can colonise the oral cavity for a short time after use and may have the potential to control oral bacterial infections only when the uptake is repeated regularly (Horz *et al.* 2007). A number of studies have reported that *L. rhamnosus* GG and two other *L. reuteri* strains can colonise the mouth of 48-100% of the study subjects consuming probiotic-containing products (Meurman *et al*. 1994; Yli-Knuuttila *et al*. 2006; Horz *et al*. 2007; Caglar *et al*. 2009) showing a variable colonising potential. It is assumed that probiotic bacteria would colonise the oral cavity only when they were used in products in contact with the mouth.

It has been observed that administration of a mixture of different *Lactobacillus* strains in capsule form causes the salivary *Lactobacillus* count to rise (Montalto *et al.* 2004). As long term colonisation of probiotics is unlikely, though possible (Yli-Knuuttila *et al.* 2006), potential adverse effects of probiotics in the oral cavity have not gained much intensive investigations. When probiotics are aimed to be used for oral health, the acidogenicity of lactobacilli and bifidobacteria cannot be ignored. For example, it has been noted that the probiotic *L. salivarius* strain is capable of inducing caries in an animal model (Matsumoto *et al.* 2005). Screening of probiotics for their effects on oral microbial communities has been recommended to allow selection of strains without a risk of oral health hazards. It has been observed that *L. salivarius* W24 has the potential to make a biofilm model more cariogenic (Pham *et al.* 2009). A significant number of studies have tested different activities of probiotics in respect of dental caries, periodontal diseases and cases of halitosis and some tumor disorders (Haukiojaa 2010; Chatterjee *et al.* 2011) showing promising results. However, most of these studies have been performed for a short time of period and, in some of them, the resulted differences were minimal, though significant in term of statistics, to build a conclusive statement for their effects. The same applied to dental caries, adding to the limitations of small samples size and short duration of follow up, it is of importance to know that the salivary level of caries associated microbes does not parallel to dental caries. Moreover, the microbiota of the whole saliva has been found to be most comparable to that of the dorsal and lateral surfaces of the tongue and microbiota of soft tissues resembles each

other more than that on the teeth and gingival margin (Mager *et al.* 2003). Therefore, no decisive declaration of the effect of probiotics on dental caries can be yet made.

1.8 Biosurfactants

Bacteria in general and probiotic in particular have the ability to produce a variety of molecules for different purposes. Numerous studies have been conducted on the properties of bacterial supernatants and their effects on other bacteria (Bermudez-Brito *et al.* 2013; Khodaii *et al.* 2017; Maslennikova *et al.* 2017). Surfactants are amphiphilic compounds that decrease the surface tension between two immiscible phases, when these substances are produced by living microorganisms such as bacteria, yeast and fungi, then they are called biosurfactants (Santos *et al.* 2016). Hence, biosurfactants are molecules of microbial origin with hydrophilic (water-soluble) and hydrophobic (oil-soluble) moieties that partition at liquid/liquid, liquid/gas or liquid/solid interfaces.

Biosurfactants have recently been used in a wide range of applications such as industrial, bioremediation, food processing, pharmaceutical, medical and oil recovery industries and others to be described as a "multifunctional biomolecules" of the 21st century (Santos *et al.* 2016). Compared to synthetic surfactants, biosurfactants have several merits such as biodegradability and lower toxicity, better environmental compatibility, higher foaming ability, higher selectivity and certain activity under hostile conditions such as extremes of temperature, pH and salinity (Shekhar *et al.* 2015).

Moreover, certain biosurfactants have been found to cause pores, destabilise biological membranes or modulate certain surface enzymes activity which make them candidates as antimicrobial, haemolytic, antiviral, antitumor, and insecticide agents. They have also been observed to regulate microbial attachment/removal from the surfaces (Vijayakumar and Saravanan 2015).

1.8.1 Biosurfactant producing microorganisms

Different microorganisms are able to produce tensioactive substances with different molecular structures and surface activities (Campos *et al.* 2013). The interest in the isolation of these biosurfactant-producing microorganisms has been increasing recently to get biological substances with good surfactant properties such as low critical micelle concentration (CMC), low toxicity and high adequate emulsifying potential. Bacteria of the

genera *Pseudomonas* and *Bacillus* have been described as great biosurfactant producers (Silva *et al.* 2014). However, due to their probable pathogenic nature, most bacteria-originated biosurfactants are not suitable and predominantly objectionable for use in food industrial formulations (Shepherd *et al*. 1995; Toribio *et al*. 2010; Saharan *et al*. 2011). On the other hand, nature does contain probiotics derived from microorganisms that generally regarded as safe (GRAS) and are recognised to manufacture a variety of biomolecules of different activities, including biosurfactants (Merk *et al.* 2005; Ceresa *et al*. 2015).

Many researchers have focussed on the future of the probiotic lactic acid bacteria (LAB) as biosurfactant-producers and their significant protagonist in term of health and industry (Sharma *et al.* 2016). For instance, the animal pathogenicity (acute oral toxicity and dermal sensitisation) test of novel xylolipid biosurfactant from *L. lactis* was performed on mice. The given doses of the biosurfactant were followed for adverse effect or lethality. No observed or measured illnesses or death were reported during the study duration and hence, the tested biosurfactant samples were placed in the toxicity category IV (i.e. safe to consume and for dermal applications) according to the FDA standards (Saravanakumari and Mani 2010).

1.8.2 Classification of biosurfactants based on the chemical nature

Biosurfactants are primarily classified based on their chemical structures. The hydrophilic moiety is usually amino acid, peptide, mono-, di-, or polysaccharide. The multiple range of properties are conferred by the structural orientation of the biosurfactants on the surfaces and inter phases which give rise to the surface and interfacial lowering ability and the formation of micelles and microemulsion (Chen *et al*. 2010; Chen *et al*. 2010a). Surface active molecules of microbial origin can be either low molecular weight which can powerfully lower the surface and interfacial tension (biosurfactant) or high molecular weight polymers that do not reduce the surface tension as much but can stabilise emulsions (bioemulsans or bioemulsifier) (Neu 1996 Rosenberg and Ron 1997; Rosenberg 2006).

1.8.2.1 Low molecular weight compounds

1.8.2.1.1 Lipopeptides

Lipopeptides are low molecular weight biosurfactant compounds. Lipopeptides can be different in the fatty acid chain and their peptide moiety and are mainly produced by *Bacillus* species (Thavasi *et al*. 2008; Soberón-Chávez 2010; Thavasi *et al*. 2011). Lipopeptides have

shown potential as antibiotics, antiviral and antitumor agents, immune-modulators or specific toxins and enzyme inhibitors.

Surfactin, a cyclic lipopeptide, is an example of a lipopeptide biosurfactant produced by *Bacillus subtilis* (Ron & Rosenberg, 2001) and is named such because of its exceptional surfactant activity (Peypoux *et al.* 1999). Surfactin has been found to lyse mammalian erythrocytes and to form spheroplasts (Bernheimer and Avigad 1970). Many bacterial species produce these biologic substances, surfactin and subtilisin are produced by *Bacillus subtilis. Bacillus licheniformis and Bacillus subtilis synthesise* Peptide-lipid and lichenysin (Yakimov *et al*. 1997; Begley *et al*. 2009), viscosin is synthesised by *Pseudomonas fluorescens* (Banat *et al*. 2010), and gramicidin is synthesised by *Bacillus brevis*, *Brevibacterium brevis* (Krauss and Chan, 1983) and many others (Shekhar *et al.* 2015). *Propionibacterium freudenreichii* subsp. *freudenreichii,* a probiotic type strain, produces a lipopeptide biosurfactant with a wide range of antimicrobial and antiadhesive potentials (Hajfarajollah *et al.* 2014)

1.8.2.1.2 Glycolipids

Biosurfactants of the glycolipid type are the most common and readily available. These are usually mono or disaccharides compounds acylated with long chain or hydroxyl fatty acids (Fracchia *et al*. 2012; Shekhar *et al*. 2015). Rhamnolipids, mannosylerythritol lipids (MELs), sophorolipids and trehalolipids are among the best studied glycolipids subclasses.

Rhamnolipids biosurfactants are produced mainly by *P. aeruginosa* and *Burkholderia*. Mono-rhamnolipids are composed of one rhamnose sugar moieties and di-rhamnolipids are composed of two, these rhamnose moiety are linked to one or two β-hydroxyfatty acid chains (Perfumo *et al*. 2006; Raza *et al*. 2009). These substances have many potential applications in the biomedical field as they display antibacterial, antifungal, antiviral and also antiadhesive effects (Remichkova *et al*. 2008; Sotirova *et al*. 2008) and have been applied in the formulation of nanoparticules (Xie *et al*. 2006; Palanisamy and Raichur 2009) and microemulsions (Xie *et al*. 2007; Nguyen and Sabatini 2009). Mannosylerythriol lipids (MELs) are synthesised by *Pseudozyma antarctica* and have recently attained interest due to their environmental compatibility, structural diversity and important variable applications in the biomedical fields. Moreover, they have been utilised in the biotechnological field for gene and drug delivery and as skin moisturisers in cosmetics (Arutchelvi and Doble 2011). *S. thermophilus* synthesises glycolipid biosurfactants with reported anti-adhesive activity

against several bacterial and yeast strains isolated from voice box prostheses (Busscher *et al*. 1997; Busscher *et al*. 1999; Rodrigues *et al*. 2004; Rodrigues *et al*. 2006). Biosurfactants produced by the probiotic *L. casei* MRTL3 and *E. faecium* have been characterised as glycolipid compound (Sharma and Singh Saharan 2014; Sharma *et al*. 2015).

1.8.2.1.3 Fatty Acids, Phospholipids and Neutral Lipids

Many yeasts, bacteria and fungi secret large quantities of phospholipids, fatty acids or neutral lipids to ease the uptake of the carbon source from the hydrophobic substrates such as alkanes (Cirigliano and Carman 1985). The hydrophilic and lipophilic balance (HLB) is directly proportional to the length of the hydrocarbon chain in their constructions. Clear microemulsions of alkanes in water were formed due to the production of phosphatidylethanolamine rich vesicles by *Acinetobacter* species (Käppeli and Finnerty 1979). Interficial tension between hexadecane and water was found to be reduced to less than 1 mN/m by bile salt synthesised by *Myroides* species (Desai and Banat 1997) and phosphatidylethanolamine synthesised by *Rhodococcus erythropolis* grown on n-alkane (Kretschmer *et al.* 1982).

1.8.2.1.4 Other biosurfactants

Viscosinamide, a cyclic depsipeptide produced by *P. fluorescens* has been reported by Nielsen and his colleagues (Nielsen *et al.* 1999) to have antifungal and surface active properties. *In-vitro* antimicrobial activity against *Mycobacterium tuberculosis* and *Mycobacterium avium-intracellulare* was attributed to two massetolides A–H, which are also cyclic depsipeptides isolated from the *Pseudomonas* spp., obtained from a marine habitat (Gerard *et al.* 1997). Sphingolipid biosurfactants degradation products have been shown to hinder the interaction of *S. mitis* with buccal epithelial cells and of *S. aureus* with nasal mucosal cells (Bibel *et al.* 1992).

1.8.2.2 High molecular weight biosurfactants

High molecular weight biosurfctants are mainly polymeric and composed of lipoproteins, proteins, polysaccharides, lipopolysaccharides or complexes containing several of these structural types (Rosenberg and Ron 1997; Rosenberg and Ron 1999; Ron and Rosenberg

2001). Alasan, liposan, lipomanan, emulsan, and some other polysaccharide–protein complexes are among the best studied polymeric biosurfactants. Emulsan has been considered as an effective emulsifying agent, even at low concentrations of 0.001 % to 0.01 %, for hydrocarbons in water (Zosim *et al.* 1982). *Candida lipolytica* has been shown to synthesise liposan as an extracellular emulsifier and *Candida tropicalis* produces mannan lipo-protein (Cirigliano and Carman 1984). *L. plantarum* has been studied for biosurfactant production and characterisation and has been documented to produce glycoprotein biosurfactant of antimicrobial activity (Madhu and Prapulla 2014).

1.8.3 Factors affecting biosurfactant production

There are many factors, beside the producer strain, that should be considered during biosurfactant production. These factors may act at the genetic, nutritional and physiochemical environment levels and can influence not only the quantity of biosurfactants produced but also the type of polymer manufactured (Hatha *et al*. 2007; Salihu *et al*. 2009).

1.8.3.1 Growth conditions

Growth conditioned media should be optimised for maximum possible gains as the production of the biosurfactants may affect by the changes of temperature, pH, and aeration or agitation status. The probiotic strain *L. lactis* CECT-4434 has been shown to produce cell-bound biosurfactants and bacteriocins when fermentation was carried out under uncontrolled pH (Rodríguez *et al.* 2010) which further emphasises the significance of pH in biosurfactants production. Extremophile-derived biosurfactants production has received interest recently for their considerable commercial opportunities. Certain biosurfactants, such as those produced by *Serratia marcescens*, are found to be able to maintain their properties at high temperature of up to 100 °C, high salt concentration (up to 12 % NaCl) and a wide pH range offering interesting and useful properties for many environmental and industrial applications. (Anyanwu *et al.* 2011). The degree of aeration and agitation is also a potential element in biosurfactant synthesis as both enable oxygen transfer from the gas phase to the aqueous phase. For instance, it has been observed that the optimum production level of the surfactant (45.5 g/L) was when the aeration was 1 vvm and the dissolved oxygen concentration was maintained at 50 % of saturation (Adamczak and Odzimierz Bednarski

2000). When aeration applied to *P. aeruginosa* LBI in a batch feed culture, the product yield increased to 70 % (Maneerat 2005). Wei et al. (2005) noticed increased rhamnolipid production up to 80 % when agitation increased from 50 to 250 rpm (Wei *et al.* 2005). Conversely, maximum biosurfactant production of the probiotic *L. plantarum* CFR occurred under stationary conditions (Madhu and Prapulla 2014). Different incubation periods of biosurfactant-producer microorganisms also have an effect on the biosurfactant production. The impact of incubation time can be assessed by monitoring the values of emulsification activity, surface tension, biomass concentration after a regular interval of time. *Pseudomonas* species, for instance, displaced the optimum rhamnolipid production of 5.86 g/L at 72 h (Soniyamby *et a.l* 2011). Other *Pseudomonas* species, *P. fluorescence*, maximised yield after 56 h (Abouseoud *et al.* 2007).

1.8.3.2 Nutritional factors

1.8.3.2.1 The effect of carbon source

Microorganisms have been shown to prefer hydrocarbons substrates over carbohydrates for biosurfactants synthesis. When microbes grow at the expense of water immiscible substrates, a burst of biosurfactant yield enhances the solubilisation of water insoluble substrates and thus assist nutrient passage to the microbes. It has also been observed that when microbes grow in media with readily available carbon source, then biosurfactant production is limited, and when the available carbon is consumed with the presence of water-immiscible hydrocarbon then biosurfactant production is triggered (Banat 1995). Rhamnolipid synthesis is regulated by the presence of n-alkane in the medium while other *Pseudomonas* species prefer vegetable oil for higher biosurfactant yield (Kosaric and sukan 2000)**.** Diesel, crude oil, glucose, sucrose, glycerol have been reported to be a good source of carbon substrate for biosurfactant production (Garima Bhardwaj *et al.* 2013).

1.8.3.2.2 The effect of nitrogen source

Rhamnolipids and sophorose lipids production was enhanced when there is a limitation of the nitrogen supply in the media (Reis *et al.* 2013). However, because nitrogen is essential for cell growth and protein and enzyme synthesis, it is considered an important element for biosurfactant manufacturing. Different nitrogen compounds have been used for the production of biosurfactants including urea peptone, yeast extract, ammonium sulphate,

ammonium nitrate, sodium nitrate, meat extract and malt extracts. Nitrate has been reported to support supreme surfactant production in *P. aeruginosa* whereas ammonium salts and urea were the preferred nitrogen sources for biosurfactant production by *Arthrobacter paraffineus* (Adamczak and odzimierz Bednarski 2000).

1.8.4 Biosurfactants in biomedicine and pharmaceutics

Biosurfactants have been proposed and used in a variety of applications in medicine and pharmaceutical formulation. The ability to form pores and destabilise biological membranes permit their use as antimicrobial, haemolytic, antiviral, antitumor, and insecticide agents (Carrillo *et al.* 2003; Scott *et al.* 2007; Ortiz *et al.* 2009). In addition, they have shown the tendency to modulate enzyme activity permitting enhancement of the activity of certain enzymes ameliorating microbial process or the inhibition of certain other enzymes permitting their use as antifungal agents (Mnif and Ghribi 2015). In addition, biosurfactants may be utilised in the field of gene therapy and gene transfection (Liu *et al*. 2010; Zhang *et al*. 2010) and respiratory failure due to the deficiency of surfactant in premature infants (Muthusamy *et al.* 2008). Biosurfactants displayed ability to aid in the recovery of intracellular products, as immunological adjuvants, in the area of tumour management as anticancer agents (Zhao *et al.* 1999) and as agents for the stimulation of skin fibroblast metabolism (Shekhar *et al.* 2015). Despite the wealth of publications concerning the biological activity of biosurfactants, their actual clinical application in the field of medicine and pharmaceuticals is still fairly narrow (Fracchia *et al.* 2015). Daptomycin, branched cyclic lipopeptide isolated from *Streptomyces roseosporus*, has been approved and commercially used as antibiotics against skin infection, endocarditis and bacteraemia caused by methicillin-resistant *Staphylococcus aureus* and other Gram-positive pathogens (Tally *et al*. 1999; Robbel and Marahiel 2010). Its mechanism of antibiotic activity was supposed to be due to membrane conformational changes and membrane pore formation (Scott *et al.* 2007).

1.8.4.1 Oral related application

The reported antimicrobial activity combined with the ability to alter substrate's surface energy and area (Busscher and Van Der Mei 1997; Lígia R. Rodrigues *et al.* 2006) for several biosurfactants would identify them as an effective candidates for oral health use. However, application of biosurfactants in oral-related health is still at an early stage. Uncharacterised glycoprotein-type biosurfactants derived from *L. casei* (ATCC39392) (Savabi *et al.* 2014), *L.*

acidophilus DSM 20079 (Tahmourespour *et al.* 2011a), *L. reuteri* (DSM20016) (Salehi *et al.* 2014) and *L. fermentum* ATCC 9338 (Tahmourespour *et al.* 2011b) have shown to intensely knockdown *S. mutans* gene expression of glucosyltransferase (gtfB and gtfC) and fructosyltransferase (ftf) genes. These genes play an important role in *S. mutans* attachment to the tooth surface which is the initial step in biofilm production and in dental caries. Gene expression down regulation in response to treatment with the biosurfactant might suggest a promising application in prophylaxis of dental caries. A partially characterised lipopeptide biosurfactant was extracted form *Bacillus tequilensis* CH and demonstrated an ability to inhibit pathogenic *S. mutans* biofilm formation on hydrophilic and hydrophobic surfaces at 50 µg/mL (Pradhan *et al.* 2013). However, the lack of evidence of complete purity or characterisation of the tested biosurfactants should be considered cautiously in interpreting these results. Though not directly involved in oral health but closely related, a glycoprotein biosurfactant was isolated from the probiotic bacteria *L. lactis* 53 and *S. thermophilus* A. Fractionation was attained by hydrophobic interaction chromatography which allowed the isolation of a fraction rich in glycoproteins. Molecular (by Fourier transform infrared spectroscopy) and elemental compositions (by X-ray photoelectron spectroscopy) were also determined. Antimicrobial activity was reported for the active fraction of the biosurfactant against orally related pathogen *S. salivarius* GB 24/9. The biosurfactant also demonstrated antiadhesive/antibiofilm properties. When artificial model voice prosthesis was preconditioned with the biosurfactant, the tested microorganisms showed a lower potential to form biofilm (Rodrigues *et al.* 2004). The finding of this study was considered promising in innovating a new strategy of prolonging the lifespan of voice prostheses. Similarly, antiadhesive activity was reported for biosurfactants (unidentified chemical structure) from *Robinia Pseudoacacia* (AC5 and AC7) and *Nerium oleander* (OC5) (Cochis *et al.* 2012) against *Candida albicans* biofilm on resins and silicon denture materials for dental prosthetic devices.

Rufino and his team (2011) described the production of the biosurfactant Rufisan from the yeast *C. lipolytica* UCP 0988. This biosurfactant showed efficacy as an antimicrobial and antibiofilm agent for a wide spectrum of pathogens, including orally related microorganisms such as *S. agalactiae*, *S. mutans*, *S. mutans* NS, *S. mutans* HG, *S. sanguis* 12, *S. oralis* J22 at a concentration superior to the biosurfactant critical micelle concentration. Although the chemical characterisation of Rufisan has not been provided in this study, the results obtained

showed that the biosurfactant from *C. lipolytica* is a potential antimicrobial and/or anti-adhesive agent for several biomedical applications (Rufino *et al.* 2011). Moreover, around 50 % and 67 % inhibition of fungal *Yarrowia lipolytica* biofilm formation on *in-vitro* 96 well plate surface and glass surface respectively, was reported when rhamnolipid was used in a pre-coating experiment. This antibiofilm potential against the opportunistic oral pathogen *Y. lipolytica* is another finding adding to the orally-related biosurfactants benefits which can further investigated to clinical application stages (Dusane *et al.* 2012). In the same framework, it has also concluded that the rhamnolipid-like biosurfactant released by the naturally oral cavity dwelling *S. mitis* can significantly discourage adhesion of *S. mutans* to a glass surface (van Hoogmoed *et al.* 2000). Using atomic force microscopy, the same research group found that coating a dental surface with *S. mitis* biosurfactant resulted in a remarkable increase in repulsion when *S. mutans* bacterial cells came close to the dental surface (van Hoogmoed *et al.* 2006). Emulsan (usually produced by *Acinetobacter calcoaceticus*) was patented as an effective antiplaque agent. Dispersion of emulsan in water and allowing it to come in contact with dental surface was shown to inhibit plaque and reduce caries. It was supposed that emulsan inhibited the adhesion of *S. mutans* through lectin-specific interaction with galactose or galactosamine on *S. mutans* cell surface. This finding suggested a possible emulsan containing toothpaste or mouthwash (Eigen *et al.* 1986), though have not been reviewed scientifically. Against these published findings related to biosurfactants effect on oral health, it has been clearly demonstrated that the application of biosurfactants in oral-related health is still at an early stage and therefore more studies are needed.

1.9 Aims and objectives

The aim of this thesis was to identify a bacteria-derived material with antimicrobial properties demonstrating a potential use in endodontics to control pulpitis and root canal infection. Bacteria-derived biosurfactants have been broadly investigated for use in a wide range of biomedical applications, however their role in endodontic infection has not been studied yet. Therefore, the main focus of this project was to investigate the potential of probiotic-derived and commercial rhamnolipid biosurfactants in the control of pulp infection.

This was achieved by:

- Investigating probiotic strains for potential antimicrobial effect against SAG and *E. faecalis*

- Determine the presence of and subsequent extracting biosurfactants.

- The physico-chemical characterisation of extracted biosurfactants.

- The proteomic analysis by mass-spectrometry to identify potential proteins of the adhesin-like type contributing to adhesion/antiadhesion effect of the biosurfactant.

- Partial purification of the extracted biosurfactant by size-exclusion chromatography.

- Evaluation of the antimicrobial/antiadhesive effects of the extracted and the commercial biosurfactants against SAG and *E. faecalis* using *in-vitro* standard antimicrobial assay and evaluating the effect of biosurfactant-coating in hampering bacterial attachment. This was enabled through the development of a method to characterise biosurfactants surface-coating.

- Elucidating the cytotoxic and apoptotic effect of the biosurfactants using pulpal fibroblasts and *ex-vivo* tooth sections alongside with quantitatively assessing their immunogenic potential on pulp tissue.

- Develop an infective co-culture tooth model to assess the *ex-vivo* antimicrobial and immune-modulatory effect of the biosurfactant.

The objective of this thesis was to introduce biosurfactants for the first time into the field of endodontic and vital pulp therapy. This will provide a substantial foundation for further investigations and development of biosurfactant-coated/containing materials to improve bacterial elimination from the infected pulp and root canal. Consequently, infections of the pulp by pathogenic bacteria are less likely to result in

the formation of abscesses and their associated complications ensuring a higher success rate of vital pulp therapy and endodontic procedure.

2 Chapter 2 Assessment of SAG/*E. faecalis* biofilm forming ability and screening of potential probiotics for antimicrobial activity against SAG/*E. faecalis*

2.1 Introduction

Dental pulp is a soft tissue enclosed within a hard rigid chamber of dentine. This protective environment is essential to keep the pulp sterile in the microbial rich niche of the oral cavity. If this secure system is disrupted for any reason, then the pulp will be in contact with pathogens directly or indirectly through bacterial toxins diffusing through dentinal tubules. Either way, the pulp responds by inflammation which is initially reversible, but which if left untreated progresses to "irreversible pulpitis" and eventually necrosis (Yu and Abbott 2007). Pulpitis is a common clinical problem and has been reported as the most frequent reason for patients seeking emergency dental care (Keenan *et al.* 2005). In cases of failure to rescue the pulp, endodontic or root canal therapy is required. These interventions attempt to eradicate bacteria from the tooth, thereby preventing further progression of infection to the deeper tissue (Levin *et al.* 2009). In general, endodontic treatment is a difficult and challenging procedure with a success rate ranging from around 65 % to over 90 % depending on clinical considerations (Levin *et al.* 2009) such as the quality of treatment and the clinical stage of pulpitis (Mukhaimer *et al.* 2012). In addition, endodontic procedures are costly and time consuming. The prevention of pulp infection and subsequent root canal treatment is therefore beneficial (Burke *et al.* 2009).

Pulpotomy involves the removal of part of the pulp which is in proximity to the stimuli while maintaining the radicular pulp. Pulpotomy procedures in both primary and permanent dentitions have variable clinical, radiological and histological success rates (Burke *et al.* 2009). The major challenge of such procedures is preventing further bacterial colonisation of the pulp.

Endogenous oral commensal bacteria have been established as the main causative agents of dental infection and pulpitis (Sundqvist 1976). A number of different microbial species have been implicated in endodontic pathology (Featherstone 2000). Members of the *Streptococcus anginosus* group (SAG) and *Enterococcus faecalis* are frequently isolated species (Molander *et al.* 1998; Pinheiro *et al.* 2003; Robertson and Smith 2009; Ledezma-Rasillo *et al.* 2010).

Whilst present in only low numbers in dental plaque, it has been proposed that SAG are important early in the pathogenic process and may actually initiate infection, thereafter preparing the environment for subsequent colonisation by anaerobic species. Interestingly, in this context, members of the SAG are often isolated in pure culture from early cases of endodontic infection (Ruviére *et al.* 2007; Robertson and Smith 2009).

Bacteriotherapy – purposeful use of bacteria or their products for the treatment of illness - (Sanders 1984) - has been studied widely as alternatives to the use of antibiotics as a response to the emergence of antibiotic resistant pathogens and the slow and expensive path of commercialising new antibiotics. Probiotics- living microorganisms that confer health benefit to the host- form the cornerstone of bacteriotherapy. The number of probiotic products entering the global market is expanding (Sanders *et al.* 2018). The majority of the probiotic health benefits have been associated with gastrointestinal diseases. More recently, considerable investigation has focussed on diverse applications of probiotics in other human body systems, mainly as antimicrobial for prevention and treatment of respiratory and urogenital infections, and as immune-modulatory agents to alleviate allergic inflammatory diseases (Della Riccia *et al.* 2007; De Vrese and Schrezenmeir 2008; Gilad *et al.* 2011; Yan *et al.* 2011). Probiotics in dental products have been proposed and studied by many. A number of studies have demonstrated the effect of probiotics on *S. mutans* in saliva as a cariogenic species (Näse *et al.* 2001; Ahola *et al.* 2002; Nikawa *et al.* 2004; Çaglar *et al.* 2005; Caglar *et al.* 2006; Caglar *et al.* 2007; Çaglar *et al.* 2008; Cildir *et al.* 2009). These studies provided promising results, however, they need to be confirmed in randomised double blind placebo studies employing larger sample populations with specific target sites in the oral cavity.

Although the mechanism of probiotics action is still not fully understood, their activities on the oral cavity can be summarised into three main mechanisms: 1- antagonising pathogens, 2- accumulation with oral microbiome and 3- modulation of the composition and consequently reduction of the pathogenicity, cariogenicity and microbial burden of the oral biofilm (Samot and Badet 2013; Schwendicke *et al.* 2014; Taheur *et al.* 2016). The other mechanism of probiotics mode of action involves interactions with the oral host tissue notably the epithelium. This interaction could enhance the defending barrier and modulate the immune response which can be utilised to competitively exclude pathogenic bacteria from their tissue binding site (Anusha *et al.* 2015; Manning *et al.* 2016).

Bacteriotherapy use in endodontics is still in its infancy. A preliminary study (Bohora and Kokate 2017) tested the *in-vitro* antagonistic efficacy of probiotic products containing species of *Lactobacillus* and *Bifidobacterium* against *E. faecalis* and *Candida albicans* as root canal pathogens. Promising antagonism was reported for the probiotics although further investigation is essential before confirming the success of application in endodontic therapy.

The number of probiotics investigated for clinical applications is expanding and broad screening may be required to select among the numerous strains. To date, no study has looked at the antimicrobial activity of probiotics against SAG as a "pioneer pathogenic bacteria" and *E. faecalis*, and possible application of probiotics or their by-products in pulp and root canal therapy. The aim of this chapter was firstly to evaluate the *in-vitro* biofilm forming capacity of the four endodontic pathogens to abiotic surface and secondly to screen five probiotic stains for their potential to inhibit the growth of the four clinical isolates. The probiotic strain with the maximum inhibitory effect would be selected for further investigation on the potential biofilm forming pathogens.

2.2 Materials and methods

2.2.1 Microbial culture conditions

Table 2.1 summarises the stock microbial isolates and media used in this study.

2.2.2 Pathogenic strains

The bacterial species used in this study (Table 2.1) were taken from the culture collection of the Oral Microbiology Unit, Cardiff School of Dentistry. These strains had been genotypically and phenotypically characterised in previous work (Everett 2018). Bacteria were grown on fastidious anaerobe agar (FAA) (Lab M™ International Diagnostic Group pic, Bury, UK) supplemented with 5 % (v/v) defibrinated horse blood (TCS Bioscience Ltd., Buckingham, UK). Brain-heart infusion (BHI) broth (Oxoid Ltd., Basingstoke, UK) was inoculated from colonies on FAA plates to prepare bacterial suspensions. *Streptococcus* spp. and *E. faecalis* were incubated in a CO_2 cabinet (5 % v/v CO_2, 20 % v/v H_2 , 70 % v/v N_2) at 37 °C (Don Whitley Scientific Ltd., Shipley, UK). *Staphylococcus aureus* NCTC8325 and *Escherichia coli* NCTC10418 were incubated aerobically at 37 °C (LTE scientific LTD, UK).

2.2.3 Probiotics

Lactococcus lactis NCTC8033, *Streptococcus thermophilus* NCTC10353 and *Lactobacillus acidophilus* NCTC12980 strains were purchased from Public Health England/NCTC and revived according to the suppliers' instructions. Briefly five mL of BHI broth was used to reconstitute the dried bacterial powder from the freeze-dried ampoule and after 5 min, a loopful was inoculated on to FAA plates and incubated at 37 °C and 5 % CO_2 for 24 h. The plates were then checked for purity and morphology of the colonies. *Lactobacillus plantarum* NCIMB8826 and *Lactobacillus fermentum* DSM20055 were kindly provided by the School of Biosciences/Cardiff University (Table 2.1).

2.2.4 Standardisation of inoculum

Overnight broth cultures of *Streptococcus* spp., *E. faecalis* RB17 and the five probiotic strains in their appropriate broth (Table 2.1) were adjusted by optical density (OD) measurements using a spectrophotometer (254 Colorimeter, Sherwood Scientific, Cambridge, UK). The equipment was calibrated at 600 nm to zero (blank) by using sterile un-inoculated broth medium. Subsequent sample readings were performed and diluted as necessary with sterile

appropriate broth to achieve a final OD (600 nm) of 0.08 – 0.1 which was found, by plating and enumeration, equivalent to approximately 1.5 x 10^8 CFU/mL.

Table 2.1 **Stock microbial isolates used and their media**

Strains	Reference source	Reference ID	Agar medium	Broth
Probiotic strains				
Lactococcus lactis subsp lactis	NCTC	8033	FAA +	BHI
Streptococcus thermophilus	NCTC	10353	M17	M17
Lactobacillus acidophilus	NCTC	12980	MRS	MRS
Lactobacillus fermentum	DSM	20055	MRS	MRS
Lactobacillus plantarum	NCIMB	8826	MRS	MRS
Pathogenic strains				
Streptococcus anginosus	Dento-alveolar abscess	670/95	FAA +	BHI
Streptococcus constellatus	Oral mucosal carcinoma	S08-07	FAA +	BHI
Streptococcus intermedius	Dento-alveolar abscess	HW13	FAA +	BHI
Enterococcus faecalis	Faecal sample	RB17	FAA +	BHI
Escherichia coli	NCTC	10418	FAA +	BHI
Staphylococcus aureus	NCTC	8325	FAA +	BHI

NCTC: National Collection of Type Cultures, DSM: German Collection of Microorganisms and Cell Cultures, NCIMB: National Collection of Industrial, Food and Marine Bacteria. FAA+: fastidious anaerobic agar supplemented with 5% defibrinated horse blood, MRS: de Man, Rogosa, Sharpe, BHI: brain heart infusion.

2.2.5 Bacterial growth curves

Bacterial suspensions were prepared as previously described (Section 2.2.4) in the appropriate broth for each strain (Table 2.1) to an OD_{600} of 0.08-0.1 (equivalent to 1.5 x 10^8 CFU/mL). Suspensions were then diluted 1:10 to give an approximate 1 x 10^7 CFU/mL. Two hundred µL of this bacterial suspension was then added to each well of a 96-well plate (SARSTEDT, Germany). Un-inoculated broth was used as a negative control. Plates were incubated within the plate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) at 37 °C, 5 % CO_2 for 24 h or 48 h depending on the strain. Growth curves were analysed according to Widdel (Widdel 2007) for the length of the lag phase (λ), the maximum growth (Amax) and the doubling time (Td) as illustrated in figure 2.1.

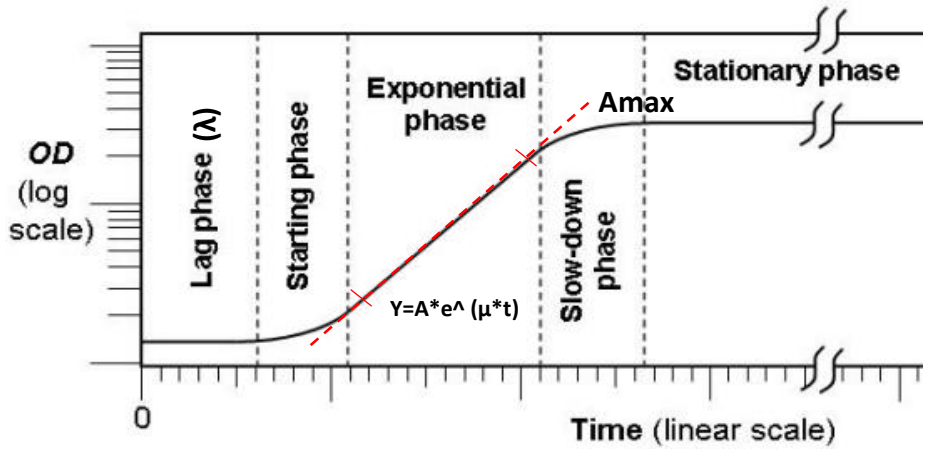


Figure 2.1 **Illustration of bacterial growth phases.** Parameters obtained from growth curves include length of lag phase (λ), maximum growth (Amax) and specific growth rate (μ). The latter was obtained from the best fit exponential equation (red dotted line). Adopted and modified from Friedrich Widdel (2007).

Doubling time was calculated according to the following equation:

$$Td = \ln 2/\mu$$

where Td is the doubling time and μ is the specific growth rate.

To find μ using excel, the log absorbance (600nm) was plotted versus time (h), and a growth curve generated. Points were selected on the exponential phase where a straight line could be drawn. A best fit equation using exponential trendline was obtained in the formula:

$$Y=A*Exp^{(\mu*t)}$$

2.2.6 Biofilm formation

This assay was performed to investigate the innate adherence and biofilm formation potential of the bacterial strains included in this study. *Streptococcus* spp., *E. faecalis* isolates and the five probiotic strains were investigated individually for adherence and biofilm formation to un-conditioned polystyrene well plate (SARSTEDT, Germany).

Overnight broth cultures of the pathogenic or probiotic strains were diluted in the appropriate broth (Table 2.1) to achieve an optical density of 0.08-0.1 at 600 nm. One further

10-fold dilution was performed. The resulting bacterial suspension was pipetted into a 6-well plate (1 mL/well). Sterile un-inoculated broth was used as a control. The lid was replaced immediately after inoculation to avoid aerial contamination and the plates were incubated at 37 °C and 5% CO_2 for 90 min to allow bacterial cells to attach. After the 90 min incubation, medium was replaced with an equal volume of new un-inoculated broth. Plates were incubated (37 °C and 5 % CO_2) for 24, 48 and 72 h with a change of medium every 24 h to remove the loosely or non-adhered bacterial cells and replenish nutrients.

At 24, 48 and 72 h incubation, the well liquid contents were aspirated gently and the wells washed gently twice with sterile phosphate-buffered saline (PBS, 1 mL/well) to remove any additional unattached bacteria. Attached bacteria were harvested with a cell scraper (25 cm, Sarstedt, USA). Crystal violet was used to stain and microscopically visualise the plate surface afterward to confirm maximal detachment of the bacterial cells from the surface. Collected bacteria were resuspended in 1mL sterile PBS in 1.5 mL centrifuge tube, vortex mixed for 10 seconds at 2500 rev/min, and diluted in 10-fold serial dilutions to 10^{-4} in sterile PBS. Four 10 µL drops of each dilution (including the original vortex mixed sample as the zero dilution) were plated according to Miles and Misra plate method (Miles *et al*. 1938) onto agar plates appropriate for the microorganism (Table 2.1). The inoculated agar plates were incubated at 5 % CO_2 and 37 °C until colonies had grown sufficiently (24-48 h) to allow visual counting of individual colonies per drop. The average count of the four drops was calculated, multiplied by the dilution factor, and then by 100 to obtain a total recovered cell count.

2.2.7 Assessing antimicrobial activity of probiotic strains

2.2.7.1 Cross streak assay

The method used by Yoshida et al. (2009) was adopted with minor modifications. Probiotic suspensions from overnight cultures in the appropriate broth (Table 2.1) were prepared and diluted to an OD_{600} of 0.08-0.1. Thick, full strength (46 g/L FAA agar and 5 % horse blood) and half strength (23 g/L FAA agar and 2.5 % defibrinated horse blood) FAA plates were prepared with the aim of representing two possible *in-vivo* conditions; nutrient rich and nutrient limited. The plates were inoculated with the prepared probiotic suspension by placing a single streak on the centre of the agar plate (the master streak) using 1 µL sterile microbiology loop (NUNC™, DENMARK). The inoculated plates were then incubated for 1, 3, 5, 7, 9 and 10 days at 37 °C and 5 % CO_2. Overnight broth of the pathogenic stains was prepared in BHI to an

OD_{600} of 0.08-0.1. These were further diluted to 1:10 in BHI. After each pre-incubation period of the streaked plates (1, 3, 5, 7, 9 and 10 days), plates were seeded with the pathogenic strains (SAG and *E. faecalis*) by a single streak at a 90° angle to the probiotic strains (but not touching the central probiotic streak) so there were 4 inhibition points with four pathogenic species examined per plate (Figure 2.2). The plates were further incubated at 37 °C and 5 % CO_2 and inspected on a daily basis for 7 days to determine the pattern of growth or interaction of the test pathogens in relation to the master streak. Negative controls were prepared using a central streak of sterile distilled water on the full and half strength FAA plates incubated at the same pre-incubation periods and inoculated with the pathogens in the same manner as the test samples. Controls were treated in exactly the same way as the test samples. Because of the difficulty to control each bacterial inoculum inoculation distance, percentage of inhibition to the total streak length was calculated:

$$\% \text{ Inhibition} = \frac{\text{Distance of inhibition (mm)}}{\text{Total cross-streak distance (mm)}} \text{ X } 100$$

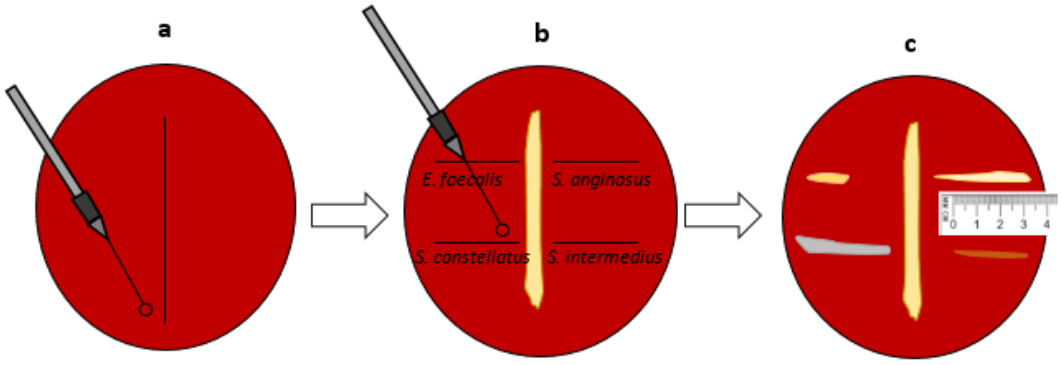


Figure 2.2 **Detection of probiotic-inhibitory effect in cross-streak experiment. a-** Line-inoculation of the antibiotic-producer strain onto the FAA Petri plate. **b-** Inoculation of the test organisms with cross-streak method just near the producer microorganism. **c-** Measurement of the inhibition distance and the total streak length after the incubation period (adopted from Tóth *et al*. 2013).

2.2.7.2 Broth inhibition assay

According to a published broth assay method (Chapman *et al.* 2013), 20 mL of 24 h broth cultures of probiotics in their appropriate broths (Table 2.1), incubated at 37 °C and 5 % CO_2 were centrifuged at 2000 *g* for 15 min (Thermo IEC CL10, UK). The resulting supernatants were filter sterilised using 0.22 µm filters (Elkay Laboratory Product LTD, UK) and their pH was measured using a pH-meter (HI2210 pH meter, ROMANIA). To account for possible acid effect, half of each supernatant was neutralised to pH 7.0 using 1 M sodium hydroxide. Neutralised and non-neutralised supernatants were filtered through 0.22 µm filter (Elkay Laboratory Product LTD, UK) and frozen in 5 mL aliquots until use. Supernatants were then defrosted at room temperature before use. SAG and *E. faecalis* were cultured overnight at 37 °C and 5 % CO_2 in BHI broth. An aliquot (5 µL) of adjusted (OD_{600}= 0.08-0.1) bacterial culture was added to 9 mL of fresh BHI broth and 1 mL of the cell-free probiotic supernatant was added to each pathogen culture. This was carried out for the pH-neutralised and the non pH-neutralised supernatants for each probiotic treatment. Growth controls were prepared by the addition of 1 mL sterile fresh probiotic broths (Table 2.1) to 9 mL sterile BHI plus 5 µL bacterial suspension aliquots. OD_{600} was measured at zero-time and the inoculated tubes were incubated immediately at 37 °C and 5 % CO_2 for 24 h. One mL of each sample was taken at 4,

8 and 24 h for OD_{600} reading, to determine inhibition of pathogen growth compared to the growth control. Gentamicin (0.1 mg/mL) was used as a positive control.

2.2.8 Statistical analysis

All experiments were performed in triplicate and each experiment repeated independently three times. Tukey's One-way analysis of variance (ANOVA) with post-hoc correction (GraphPad InStat 3 (v3.06)) was used to analyse statistical difference between test groups' means of biofilm assay. Dunnett's mean comparison one-way ANOVA was used with the broth inhibitory assay. A p value of ≤ 0.05 was considered significant (*, $p < 0.05$), very significant (**, $p < 0.01$) or highly significant (***, $p < 0.001$).

2.3 Results

2.3.1 Bacterial growth in various culture media

The growth curves of probiotic strains and pathogenic clinical isolates in the appropriate culture media are presented in figure 2.3 and figure 2.4. Table 2.2 depicts growth curve parameters for the pathogenic strains and the probiotic strains.

All clinical isolates were grown in BHI at 37 °C and 5 % CO_2. Their growth curves (Figure 2.3) followed a typical bacterial growth pattern demonstrated by the initial lag phase, followed by exponential growth and stationery phase when the nutrients may be inadequate. Although the starting inoculum concentration was the same for all strains, parameters (Table 2.2) of the growth phase for the different strains were shown to differ. The shortest lag phase was observed for *E. faecalis* (λ = 1.34 ± 0.5) and it reached the stationary phase earlier than the other stains (5 h compared to 9, 8 and 15 h for *S. anginosus*, *S. intermedius* and *S. constellatus* respectively) with a short duplication time (Td) of 0.63 ± 0.3 h. *S. anginosus* had a maximum absorbance (Amax) comparable to that of *E. faecalis* (0.83 ± 0.015 and 0.84 ± 0.03 respectively), however it appeared to replicate more slowly than *E. faecalis* (Td = 1.34 ± 0.16 h). *S. constellatus* demonstrated the longest lag phase of 4 ± 1 h and the longest Td of 2.3 ± 0.24 h. *S. intermedius* required less time to enter the exponential growth phase (λ = 2.6 ± 0.5 h) and was faster to duplicate (Td= 0.82 ± 0.15 h) in comparison to S*. constellatu*s: However, the Amax of these strains was comparable (0.65 ± 0.07 and 0.56 ± 0.03 respectively).

Regarding the probiotic strains (Figure 2.4, Table 2.2), *L. lactis* grew well in BHI broth and on FAA plate but did not grow well on de Man, Rogosa, and Sharp (MRS) agar medium; the former two media were selected for growth of *L. lactis* in broth and agar culture, respectively. *L. plantarum* demonstrated the highest Amax of 1.85 ± 0.065 followed by *L. fermentum* (Amax = 1.37 ± 0.09) and *L. lactis* (0.74 ± 0.035). Although *L. plantarum* and *S. thermophilus* entered exponential growth at comparable times (λ= 4.16 ± 0.28 and 4.5 ± 1.8 respectively) and had comparable growth rates (Td = 1.45 ± 0.18 and 1.57 ± 0.25 respectively), *L. plantarum* showed the highest Amax (1.85 ± 0.065) while *S. thermophilus* showed the lowest Amax (0.29 ± 0.05). *L. acidophilus* required 8 ± 1 h for signs of replication to be detected (Td= 4.2 ± 1.66) and appeared to reach stationary phase after around 30 h, therefore incubation was continued for 48 h.

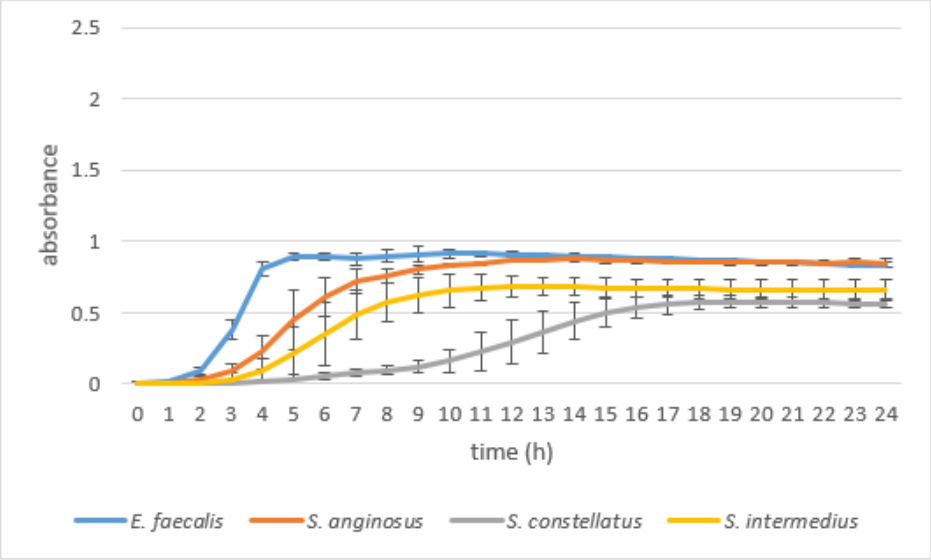


Figure 2.3 **SAG and *E. faecalis* isolates growth curve**. Absorbance of bacterial suspension grown in BHI broth, showing bacterial growth phases for 24 hours incubation at 37°C, 5% CO2. Error bars represent the standard error of the mean (SEM) of three independent experiments.

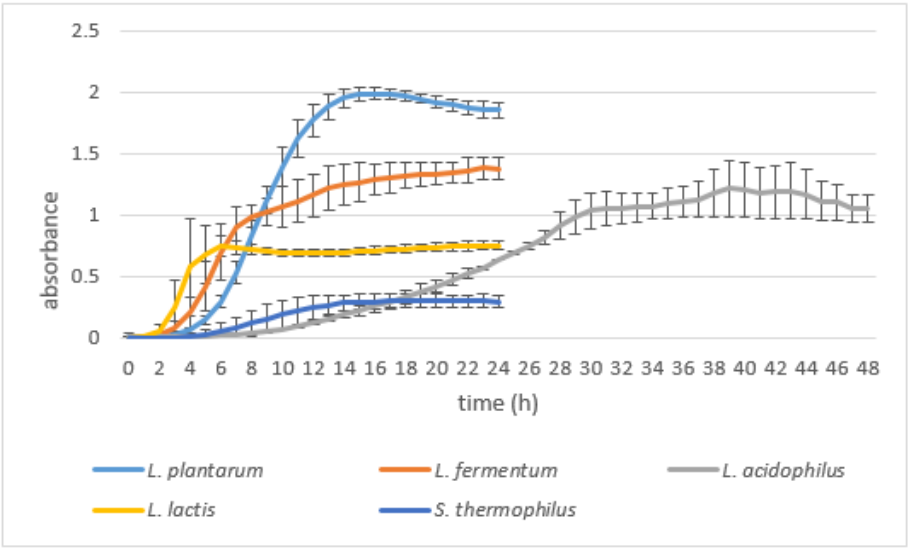


Figure 2.4 **Probiotic strains growth curve**. Absorbance of bacterial suspension grown in MRS broth for *L. plantarum*, *L. fermentum* and *L. acidophilus*, BHI broth for *L. lactis* and M17 broth for *S. thermophilus*, showing bacterial growth phases and total growth of isolates after 24 hours incubation at 37°C, 5% CO_2. Error bars represent standard error of the mean.

Table 2.2 **Pathogenic and probiotic growth parameters.**

Strains	Amax	length of lag phase (λ)	doubling time Td (h)
S. anginosus	0.83 ± 0.015	3 ± 0.2	1.34 ± 0.16
S. constellatus	0.56 ± 0.03	4 ± 1	2.3 ± 0.24
S. intermedius	0.65 ± 0.07	2.6 ± 0.5	0.82 ± 0.15
E. faecalis	0.84 ± 0.03	1.34 ± 0.5	0.63 ± 0.3
L. lactis	0.74 ± 0.035	2 ± 0.3	0.84± 0.10
L. plantarum	1.85 ± 0.065	4.16 ± 0.28	1.45 ± 0.18
L. fermentum	1.37 ± 0.09	2. 4 ± 0.28	0.95 ± 0.20
***L. acidophilus* 24h**	0.63 ± 0.10	-	-
***L. acidophilus* 48h**	1.05 ± 0.23	8 ± 1	4.2 ± 1.66
S. thermophilus	0.29 ± 0.05	4.5 ± 1.8	1.57 ± 0.25

2.3.2 Biofilm Assay

The potential of the pathogenic and probiotic strains to adhere to and maintain viability on microtitre plates was determined and used to infer the potential of these strains to develop into biofilms (Figure 2.5 and Figure 2.6 respectively). Recovery and subsequent plating and enumeration of the attached viable cell after 24, 48 and 72 h was performed. This method was used to quantify the bacterial cells (CFU/mL) in the biofilm biomass (Djordjevic *et al.* 2002; Azeredo *et al.* 2017).

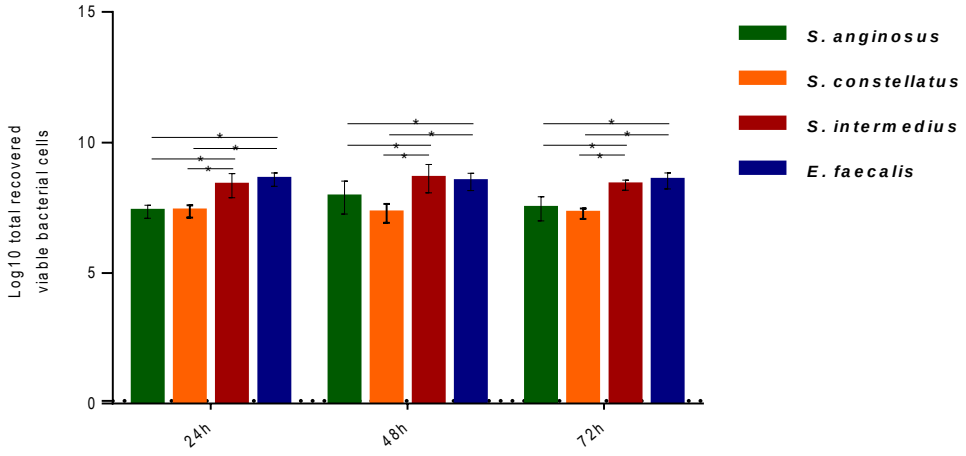


Figure 2.5 **Comparison of biofilm forming-capacity between the four pathogenic strains**. The propensity of each pathogen to form biofilms was measured after 24 h, 48 h and 72 h culture in a 6-well polystyrene plates by plating and enumeration on FAA plate. Data are expressed as the mean ± SEM. * = $p \leq 0.05$ versus all strains was considered significant using Tukey's one way ANOVA.

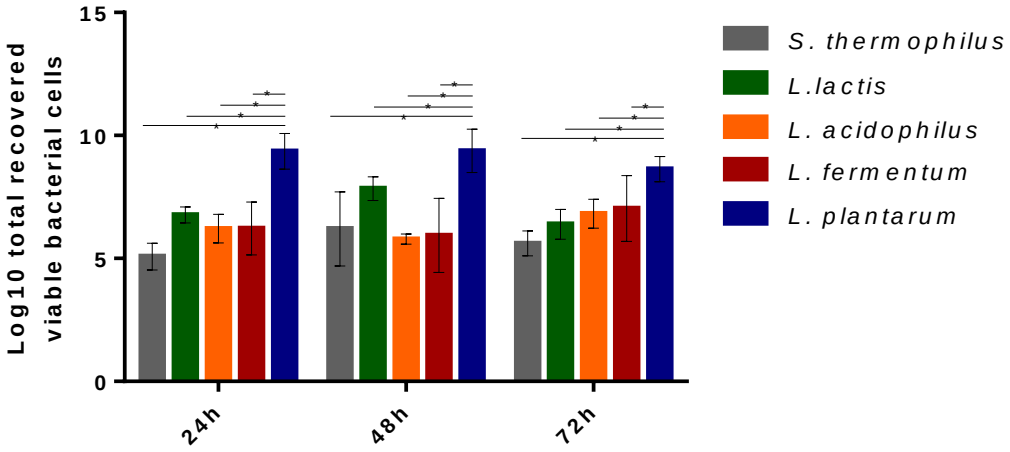


Figure 2.6 **Comparison of biofilm forming-capacity between the five probiotic strains**. The propensity of each probiotic strain to form biofilms was measured after 24, 48 and 72h culture in a 6-well polystyrene plates by enumeration on appropriate media. Data are expressed as the mean ± SEM. * = $p \leq 0.05$ versus all strains was considered significant using Tukey's one way ANOVA.

Each strain of the four pathogenic strains demonstrated the ability to form consistent biofilm over 24, 48 and 72 h as shown statistically by no significant difference ($p \geq 0.05$) of the recovered viable cells over these time periods (Figure 2.5). No significant difference was encountered in the number of viable cells recovered from *E. faecalis* and *S. intermedius* biofilm over 24, 48 and 72 h ($p > 0.05$) but significantly ($p \leq 0.05$) more viable bacterial cells were recovered from both of these strains in comparison to *S. anginosus* and *S. constellatus* biofilm. The two latter strains formed 24, 48 and 72 h-old biofilms with comparable ($p > 0.05$) biomass (as measured by the recovered viable cells count).

Figure 2.6 compares the biofilm propensity of the five probiotic strains over period of 24, 48 and 72 h. As with the pathogenic strains, plating and counting of the detached bacterial population was used to assess biofilm formation. No significant difference ($p > 0.05$) was detected between 24, 48 and 72 h-old biofilm of each of the probiotic strains. However, the number of viable cells recovered from *L. plantarum* biofilm grown for 24, 48 and 72 h was significantly ($p \leq 0.05$) higher than that of the other probiotic strains biofilms grown for the corresponding time period. *S. thermophilus*, on the other hand, formed a 24 h-old biofilm with the least number of viable cells ($\log_{10} = 5.07 \pm 0.54$) and increased slightly but not significantly over the following 24 h ($\log_{10} = 6.2 \pm 1.5$) to drop again in the 72 h biofilm culture ($\log_{10}$ 5.6 ± 0.50).

2.3.3 *In-vitro* screening probiotics for their antimicrobial effects

2.3.3.1 Cross streak assay

The indicator strains were pre-incubated for 1, 3, 5, 7, 9, and 10 days. However, only three time periods (1, 7 and 10 days) are presented here as they showed the most marked and typical differences. As shown in figure 2.7 and table 2.3, out of the five probiotic culture streaked as indicator strains, *L. acidophilus* and *S. thermophilus* did not affect the growth of any of SAG or *E. faecalis* (% of inhibition = 0) at any of the pre-incubation periods inspected.

Pre-incubation period was found to have a substantial effect on the appearance of the inhibitory effect of the other three probiotic strains (*L. plantarum*, *L. fermentum* and *L. lactis*). Using full strength FAA plate, a pre-incubation period of 7 days was required for *L. plantarum* to inhibit the growth of *S. anginosus* by 27.1 ± 1.4 %, *S. constellatus* by 37.2 ± 5.7 and *S.*

intermedius by 69.84 ± 3.9 % (Figure 2.7, Table 2.3). A longer *L. plantarum* pre-incubation of 10 days resulted in 100 % inhibition of the three SAG members indicating a concentration-dependent effect (Figure 2.7, Table 2.3). Although *E. faecalis* did not demonstrate any zone of inhibition, its growth was remarkably constricted when grown with the 10 d pre-incubated *L. plantarum* (Figure 2.8) when compared to the negative control plate. Comparable results were obtained when *L. plantarum* was inoculated on half-strength FAA plates (Figure 2.7, Table 2.3). 100 % SAG growth inhibition was observed at 10 d pre-incubation. However, 7 d pre-incubation caused inhibition of only *S. constellatus* (35.5 % ± 3.6) and *S. intermedius* (32.39 % ± 3.45) but not of *S. anginosus*. No inhibition of *E. faecalis* was recorded at any time on the half-strength FAA plate (Figure 2.7, Table 2.3).

L. fermentum did not show any antimicrobial effect when cultured on half-strength FAA plates even after prolonged pre-incubation of 10 d. However, a profound anti-SAG activity (100 % inhibition) was found when *L. fermentum* was incubated for 10 d on full-strength FAA plates before SAG were cross streaked on. A shorter pre-incubation period of 7 d caused 15.05 % ± 4.06 inhibition of *S. constellatus* and 11.17 % ± 4.74 inhibition of *S. intermedius* but not of *S. anginosus. E. faecalis* was found resistant to the antimicrobial effect of *L. fermentum.*

As with *L. fermentum*, *L. lactis* was not able to impede bacterial growth of the tester strains on half-strength FAA plates, however, 100 % inhibition of *S. constellatus* and *S. intermedius* was reported on full-strength 10 d pre-incubation plate. A shorter pre-incubation of 7 d resulted in much less inhibition of *S. constellatus* and *S. intermedius* (14.46 % ± 3.53 and 13.12 ± 3.09 respectively).

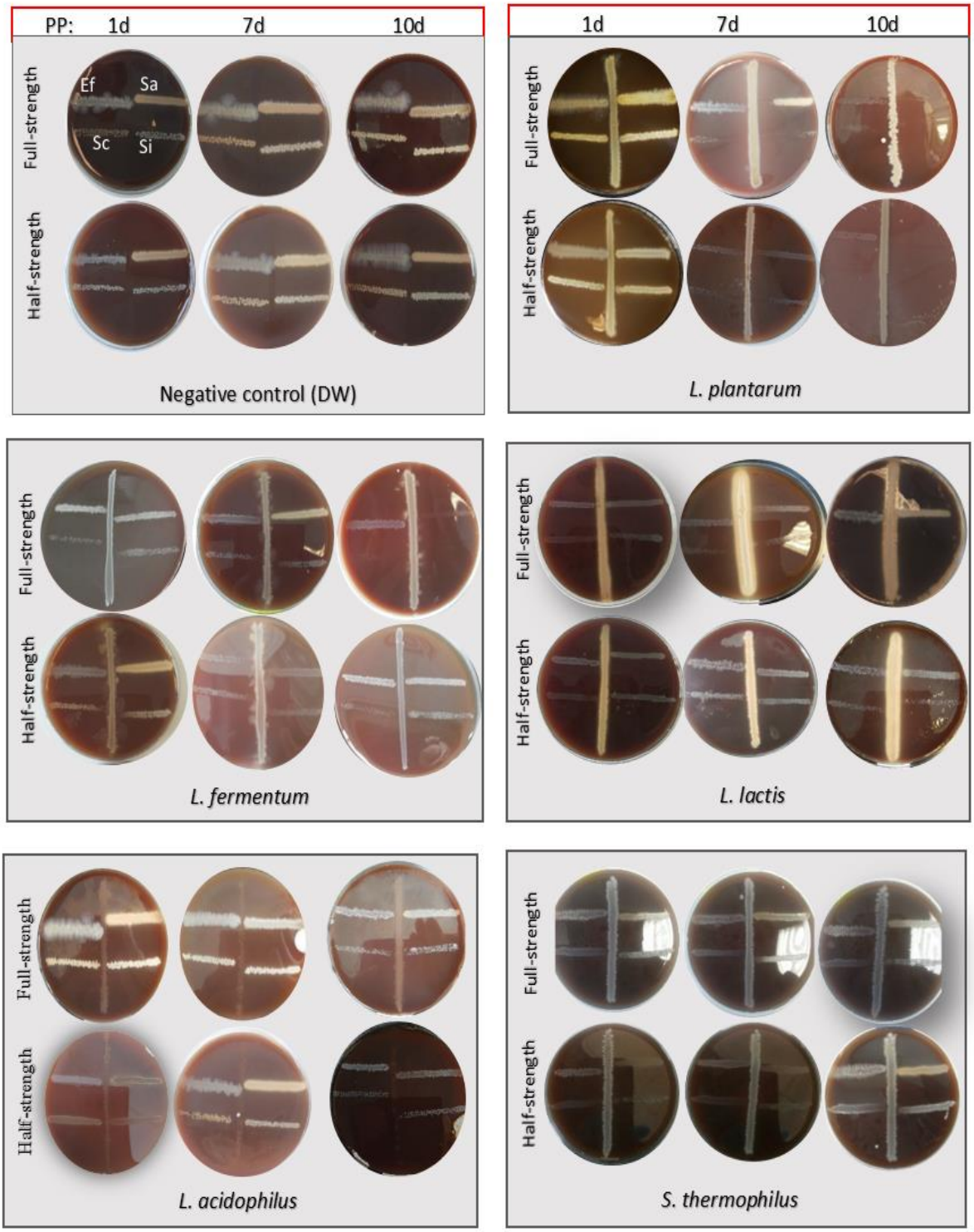


Figure 2.7 **Cross streak assay.** Centrally streaked probiotic stain (sterile distilled water was used as the control) was pre-incubated for 1, 7, and 10 days on full and half-strength FAA plates. After co-culturing and incubation, inhibition and total streak distance was measured by ruler. Ef, Sa, Si and Sc stand for *E. faecalis*, *S. anginosus*, *S. intermedius* and *S. constellatus* respectively. PP: pre-incubation period. Note: all plates were at the same orientation of the Ef, Sa, Sc, Si-labelled top left corner one. The experiment was performed in triplicate on three different occasions.

Figure 2.8 ***E. faecalis*** **growth difference between the negative control (left red arrow) and** ***L. plantarum*** **pre-incubated for 10 days (right red arrow).** Note: the plates were left 3 days after co-culturing.

Table 2.3 **Percentage zone of inhibition in cross streak assay**. Data presented as mean ± SEM (n=3). Zero indicates there was no observed inhibition of the pathogenic streaks. 100% represents a complete inhibition of the total pathogenic streaks.

		% inhibited distance to the total streak distance											
		Full strength FAA plate						**Half strength FAA plate**					
		1d		**7d**		**10d**		**1d**		**7d**		**10d**	
probiotic strain	**pathogen strain**	**mean**	**SEM**	**mean**	**SEM**	**mean**	**SEM**	**mean**	**SEM**	**mean**	**SEM**	**mean**	**SEM**
L. plantarum	**Ef**	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0
	Sa	0	± 0	27.1	± 1.43	100	± 0	0	± 0	0	± 0	100	± 0
	Sc	0	± 0	37.26	± 5.71	100	± 0	0	± 0	35.5	± 3.6	100	± 0
	Si	0	± 0	69.84	± 3.96	100	± 0	0	± 0	32.39	± 3.45	100	± 0
L. fermentum	**Ef**	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0
	Sa	0	± 0	0	± 0	100	± 0	0	± 0	0	± 0	0	± 0
	Sc	0	± 0	15.05	± 4.06	100	± 0	0	± 0	0	± 0	0	± 0
	Si	0	± 0	11.17	± 4.74	100	± 0	0	± 0	0	± 0	0	± 0
L. lactis	**Ef**	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0
	Sa	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0
	Sc	0	± 0	14.46	± 3.53	100	± 0	0	± 0	0	± 0	0	± 0
	Si	0	± 0	13.12	± 3.09	100	± 0	0	± 0	0	± 0	0	± 0
L. acidophilus	**Ef**	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0
	Sa	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0
	Sc	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0
	Si	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0
S. thermophilus	**Ef**	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0
	Sa	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0
	Sc	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0
	Si	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0	0	± 0

Ef, *E. faecalis; Sa, S. anginosus; Sc, S. constellatus; Si, S. intermedius*; d, day; SEM, standard error of the mean.

2.3.3.2 Broth inhibition assay

To determine whether there was inhibitory activity of the excreted by-products from the probiotic strains against the tested endodontic pathogens, a broth inhibitory assay was undertaken. Test samples growth patterns were followed for 0, 4, 8 and 24 h by measuring the absorbance (600 nm) at each time point and comparing it to the untreated growth control. It was found that all the probiotic supernatants were acidic (Table 2.4). While *L. plantarum* supernatant was the most acidic (pH = 4.0 ± 0.3), *S. thermophilus* supernatant was found to produce the least acidic supernatant (pH = 6.5 ± 0.1) so did not need pH adjustment. The lactobacilli strains were grown in MRS broth, *L. lactis* in BHI and *S. thermophilus* in M17.

2.3.3.2.1 Testing against *E. faecalis* growth

It was observed, from the turbidity reading, that *E. faecalis* growth was significantly ($p \leq 0.05$) reduced at 8 and 24 h time points when mixed with non-pH-controlled supernatants of *L. acidophilus*, *L. fermentum* and *L. plantarum*. However, upon pH neutralisation of the three probiotic supernatants to pH 7.0, no significant ($p > 0.05$) growth reduction (as measured by the absorbance reading) was reported for *E. faecalis* at 8 and 24 h incubation (Figure 2.9a). *L. lactis* and *S. thermophilus* supernatants were found to have no effect on the *Enterococcus* growth demonstrated by no significant ($p > 0.05$) difference in the absorbance of the test samples and the control (Figure 2.9b and Figure 2.9c). These comparisons were undertaken against the untreated growth control (negative control).

2.3.3.2.2 Testing against *S. anginosus* growth

As shown in figure 2.10a, b and c, only *L. fermentum* and *L. plantarum* supernatants (pH-controlled and non-pH-controlled) at 24 h caused a significant ($p \leq 0.01$) *S. anginosus* growth reduction in comparison to the untreated growth control (negative control).

2.3.3.2.3 Testing against *S. constellatus* growth

Although statistically not-significant ($p > 0.05$), the non-pH-controlled supernatants of the three probiotic lactobacilli appeared to reduce the growth of *S. constellatus* at 24 h incubation. Upon pH adjustment *L. plantarum* and *L. acidophilus* supernatants inhibited *S. constellatus* growth though again not significantly ($p > 0.05$) as shown in figure 2.11a. No effect ($p > 0.05$) was noticed in co-culture with *L. lactis* and *S. thermophilus* supernatants (Figure 2.11b and Figure 2.11c).

2.3.3.2.4 Testing against *S. intermedius* growth

After 24 h incubation, significant reduction ($p \leq 0.05$) of *S. intermedius* growth was observed following treatment with the pH-controlled and non-pH-controlled supernatants of *L. acidophilus*, *L. fermentum* and *L. plantarum* (Figure 2.12a) in comparison to the untreated negative growth control. No inhibitory effect of *L. lactis* supernatant (Figure 2.12b) and *S. thermophilus* (Figure 2.12c) were observed against *S. intermedius*.

Table 2.4 **pH reading of 24 h-probiotic supernatant indicating acid production.** Data is expressed as mean ± standard deviation (n=3).

Probiotic	Culture broth/pH	24 h-probiotic supernatant pH
L. acidophilus	MRS/6.2 ± 0.3	4.3 ± 0.2
L. plantarum		4.0 ± 0.3
L. fermentum		4.3 ± 0.4
L. lactis	BHI/7.4 ± 0.2	5.0 ± 0.2
S. thermophilus	M17/6.9 ± 0.2	6.5 ± 0.1

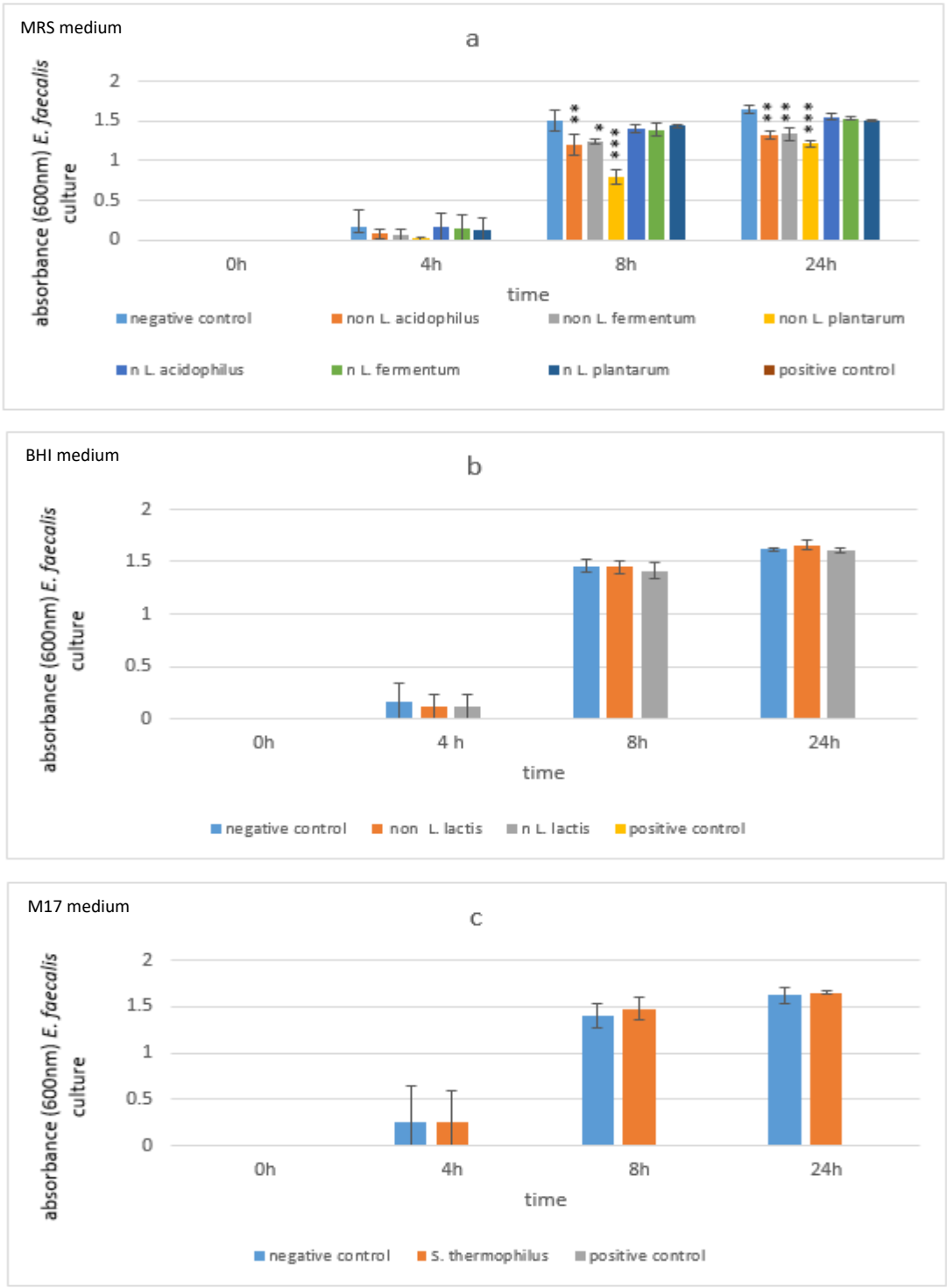


Figure 2.9 ***E. faecalis*** **growth patterns** following treatment with **a-** pH-controlled and non-pH controlled *L. acidophilus*, *L. fermentum* and *L. plantarum* supernatants using MRS broth supernatant. **b.** pH-controlled and non-pH controlled *L. lactis* supernatant in BHI broth, **c.** *S. thermophilus* supernatant using M17 broth. Data are expressed as mean ± SEM. n, pH-neutralised; non, non-pH neutralised; negative control, untreated growth control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ using Dunnett's one-way ANOVA.

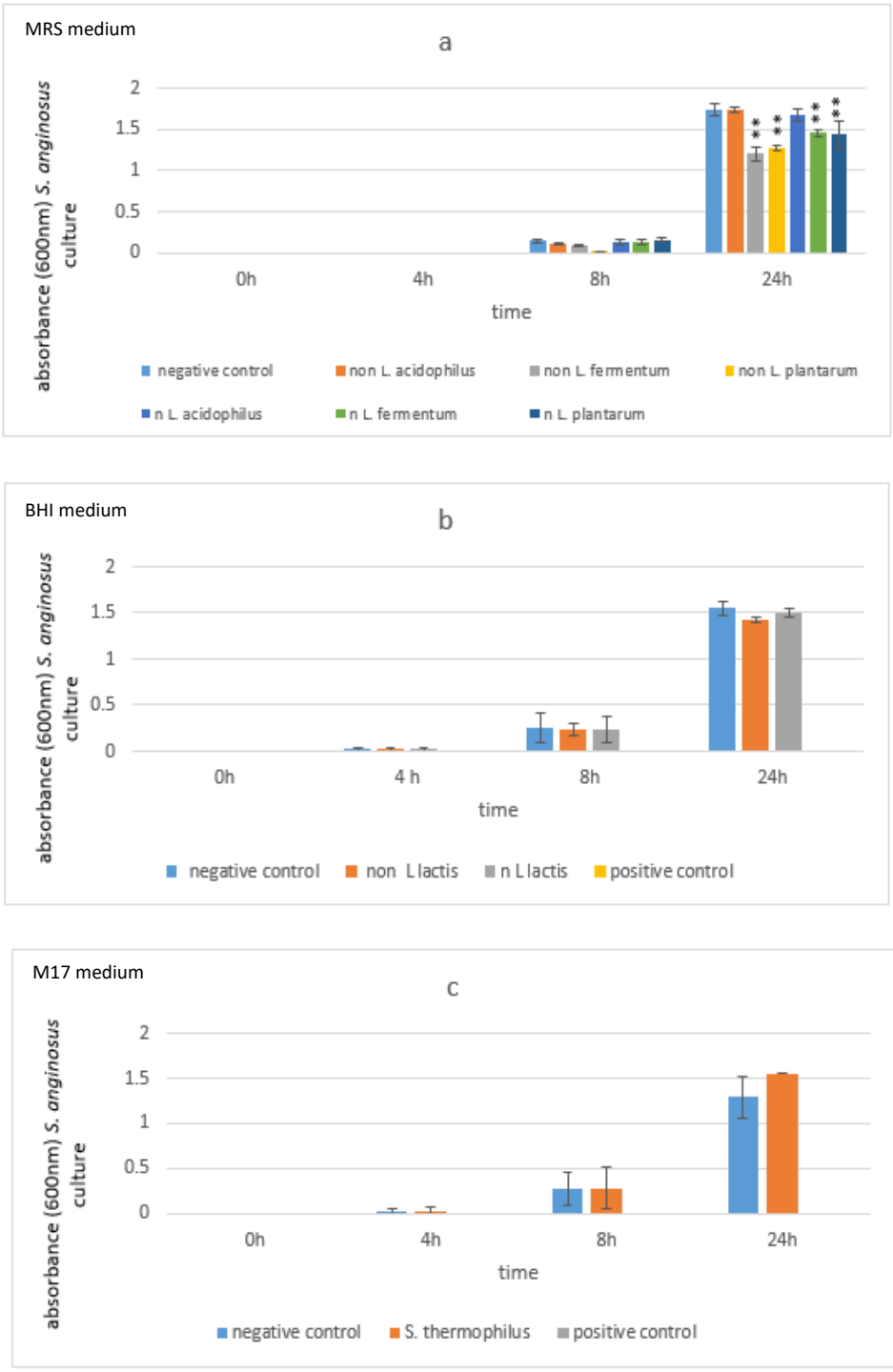


Figure 2.10 ***S. anginosus*** **growth patterns** following treatment with **a-** pH-controlled and non-pH controlled *L. acidophilus*, *L. fermentum* and *L. plantarum* supernatants using MRS broth supernatant. **b.** pH-controlled and non-pH controlled *L. lactis* supernatant in BHI broth, **c.** *S. thermophius* supernatant using M17 broth. Data are expressed as mean ± SEM. n, pH-neutralised; non, non-pH neutralised; negative control, untreated growth control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ using Dunnett's one way ANOVA.

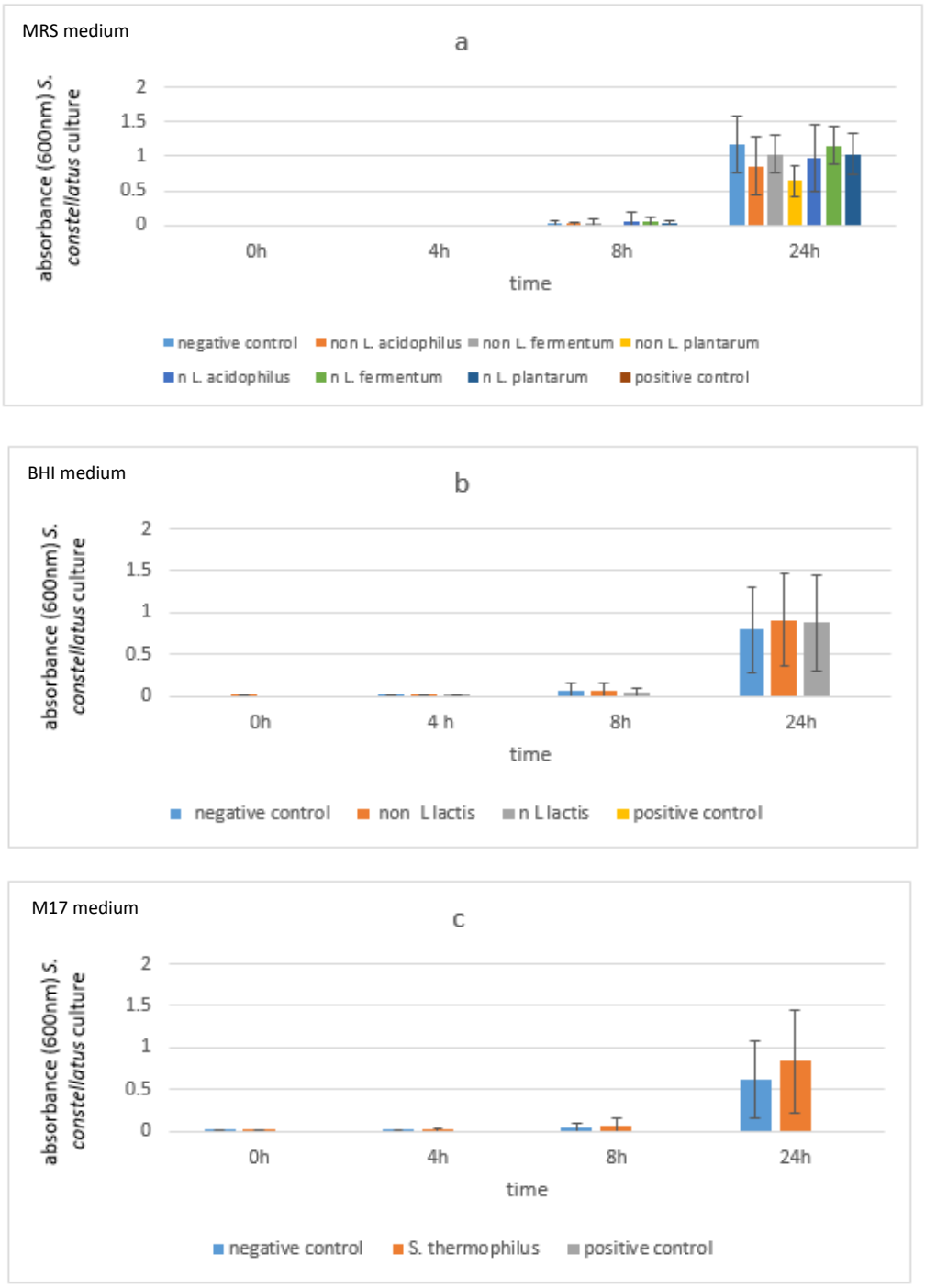


Figure 2.11 ***S. constellatus*** **growth curve patterns** following treatment with **a-** pH-controlled and non-pH controlled *L. acidophilus*, *L. fermentum* and *L. plantarum* supernatants using MRS broth supernatant. **b.** pH-controlled and non-pH controlled *L. lactis* supernatant in BHI broth, **c.** *S. thermophius* supernatant using M17 broth. Data are expressed as mean ± SEM. n, pH-neutralised; non, non-pH neutralised; negative control, untreated growth control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ using Dunnett's one-way ANOVA.

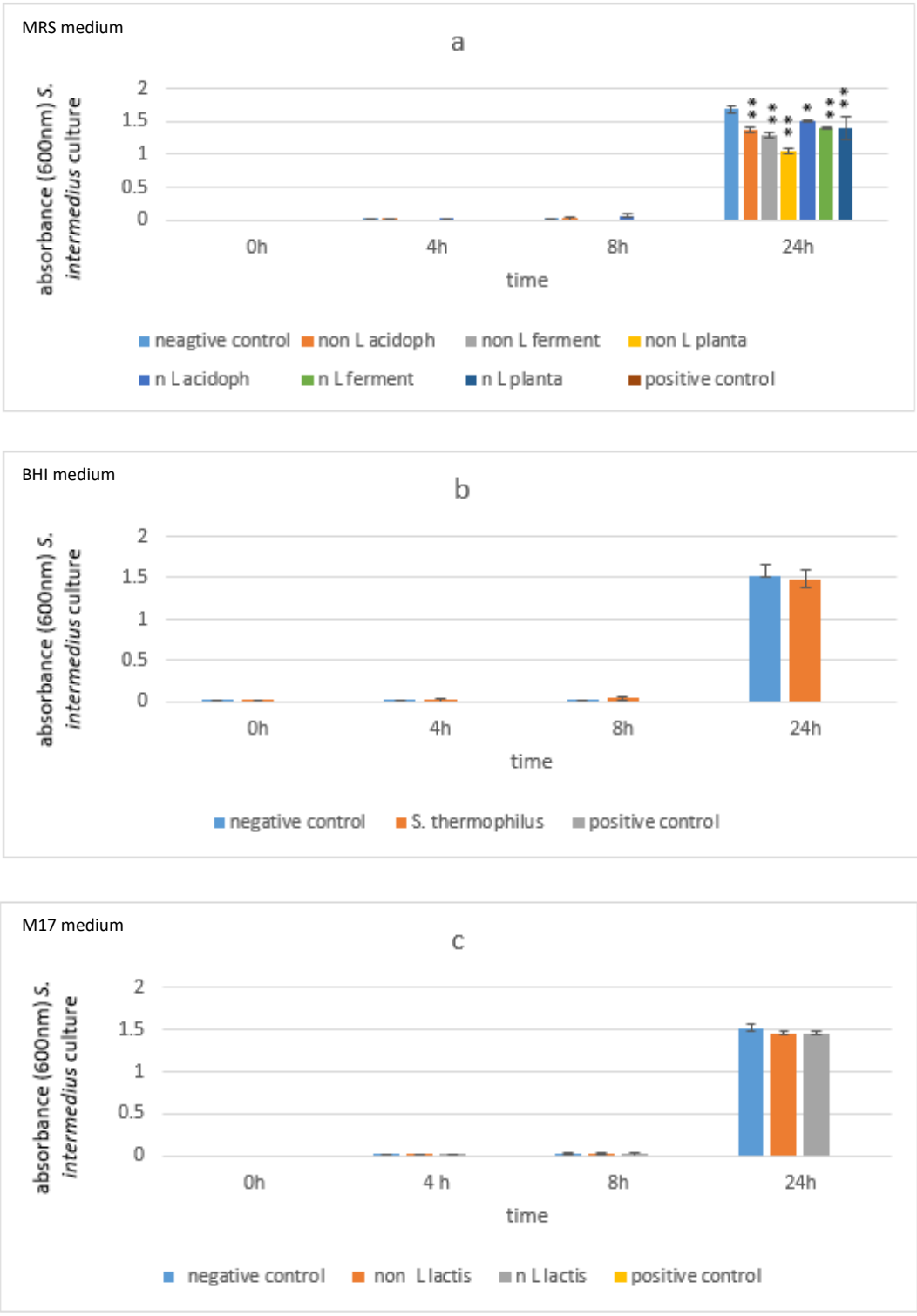


Figure 2.12 ***S. intermedius* growth curve patterns** following treatment with **a-** pH-controlled and non-pH controlled *L. acidophilus*, *L. fermentum* and *L. plantarum* supernatants using MRS broth supernatant. **b.** pH-controlled and non-pH controlled *L. lactis* supernatant in BHI broth, **c.** *S. thermophius* supernatant using M17 broth. Data are expressed as mean ± SEM. n, pH-neutralised; non, non-pH neutralised; negative control, untreated growth control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ using Dunnett's one-way ANOVA.

2.4 Discussion

This chapter presents a novel *in-vitro* comparison of the antimicrobial capacity of five probiotic bacterial strains against four potential endodontic pathogens. Overall, *L. plantarum* showed the most inhibitory activity against SAG members and *E. faecalis*; activity was demonstrated both for cells growing on solid agar and within broth culture. *L. fermentum* exhibited comparable anti-SAG activity to that of *L. plantarum* but only on full strength FAA medium. *S. thermophilus*, on the other hand, did not show any antimicrobial ability. These results led to the selection of *L. plantarum* from the five studied probiotics as the most promising species for further investigation in the following chapters. The results also highlighted that the *E. faecalis* strain studied was the most resistant to inhibition by the current study methods.

Additional analysis revealed that the four pathogenic strains showed the ability to adhere and increase in cell number on the plastic surface of the well plate; a widely employed indicator of good biofilm forming capacity. *E. faecalis* and *S. intermedius* both demonstrated a good biofilm forming capacity with the two highest recovered viable cell count (Figure 2.5). These findings will be employed later to study the antiadhesive effect of *L. plantarum* extracts on SAG and *E. faecalis* attachment.

Despite the wide range of currently available endodontic and vital pulp therapies, there are still considerable high failure rates (Bernstein *et al.* 2012) which necessitates further clinic visits and costs and may lead to more invasive therapy or ultimately tooth extraction. One important factor associated with failure is the continued presence of bacteria within the pulp and root canal system (Siqueira 2001; Nair 2006). According to one review (Jhajharia *et al.* 2015), endodontic infection is mostly a surface-associated microbial invasion and the term “biofilm” should be considered when dealing with such infection. Eradication of this form of bacterial burden is a crucial requirement of successful treatment (Sundqvist *et al.* 1998).

Probiotic bacteria and their by-products have been studied widely for their antimicrobial and immune-modulatory effects in different human body systems (Kechagia *et al.* 2013). It has been hypothesised in the current study that these activities can be harnessed for dental application to treat or prevent pulpitis and subsequent endodontic infection.

The three “anginosus” group members (*S. anginosus*, *S. constellatus* and *S. intermedius*) and *E. faecalis* were selected for the current study as they are considered part of the commensal oral microbiota but also associated with pulpitis and endodontic infections. The “anginosus” group has been reported to associated with about one third of endodontic infections (Siqueira and Rôças 2009) and *E. faecalis* has been frequently isolated from failed endodontic and apical periodontitis (Portenier *et al.* 2003; Ozbek *et al.* 2009).

Although the studied strains belong to the same group, their biofilm formation potential was found here to be different confirming previous reports of considerable phenotypic and genotypic heterogeneity within the group. *S. intermedius* and *E. faecalis* appeared to show the greatest biofilm forming potential, as measured by the recovered viable cells followed by *S. anginosus* and *S. constellatus*. This might be attributed to the biofilm-phenotypic characterisation. Only a limited number of publications are available regarding SAG biofilm-phenotypic characterisation (Eick *et al*. 2004; Fernanda Petersen *et al*. 2004; Landrygan-Bakri *et al*. 2012; Takeshi Yamanaka 2013). By employing the matrix proteoglycans of decorin and biglycan of soft gingival and alveolar bone to assess real-time SAGs adherence and biofilm formation, Landrygan-Bakri et al. (2012) found a significant ability of *S. constellatus* and *S. intermedius* strains to adhere to the glycosaminoglycan (GAG) moiety of the extracellular matrix proteoglycans decorin and biglycan. The possession of high chondroitinase activity by selected isolates of *S. constellatus* and *S. intermedius* (322/95, 34C, 127/95, 30C) and its absence from the more poorly-adhering isolates of *S. anginosus* was proposed by the author to be critical for the release of the GAG chain from the mother molecule and for subsequent interactions. The phenotype/genotype characteristic of the bacterial cell may be influenced by such interaction thus determining whether or not “commensal” organisms initiate an infective process. Moreover, *S. constellatus* and *S. intermedius* adherence to such surface proteins of alveolar bone may enhance their survival and potential virulence in the periapical region lending the tissue susceptible to infective biofilm and abscess formation.

Petersen et al. (2004) emphasised the role of quorum sensing in encouraging a biofilm mode of growth of *S. intermedius* NCTC 11324. They reported that a 24 h incubation of *S. intermedius* with the addition of synthetic competence-stimulating signalling peptide (CSP) at 5 % CO_2 and 37 °C, resulted in a 100 % increase in biofilm formation without affecting growth rate when compared to CSP-untreated growth.

In contrast, a study conducted by Yamanaka et al. (2013) examined the *in-vitro* biofilm forming capacity of one clinical *S. constellatus* strain (designated as H39) isolated from a lesion resulting from infection of the root canal and three reference SAG strains (*S. constellatus* ATCC 27823, *S. intermedius* ATCC 27335 and *S. anginosus* ATCC 33397). Biofilm phenotype of the clinical strain H39 and the type strains of SAG was assayed via a crystal violet staining microtiter plate method. *S. constellatus* strain H39 succeeded in forming consistent biofilm which was comparable to that of the species reference strain followed by *S. intermedius* ATCC 27335. While *S. anginosus* ATCC 33397 failed to grow as biofilm. Scanning electron microscopy (SEM) images of the grown biofilms showed that the clinical and reference strains of *S. constellatus* were able to produce a dense meshwork-like structure around the cell surface but other strains of *S. anginosus* ATCC 33397 and *S. intermedius* ATCC 27335 lacked this phenotype. Moreover, the two strains of *S. constellatus* were found to produce a highly viscous material in the spent culture medium compared to the un-inoculated broth. However, no change in the viscosity was reported for *S. intermedius* and *S. anginosus* spent culture medium. These findings might explain, to some extent, the different biofilm potential amongst the different strains of the same group. Of further consideration, biofilm formation is considered an important step in initiating host response to infection and may contribute to pathogenicity of that phenotype (Parsek and Singh 2003). Hence, the current finding that different SAG members differ in their biofilm forming potential might affect their pathogenicity and tropism in pulp and endodontic infections. In 2001, Claridge and colleagues conducted a study that aimed to assess the clinical importance of individual species of the "*Streptococcus milleri* group" (SMG) and abscess formation. SMG isolates had been identified to species level in 118 clinical cases using 16S rRNA sequence analysis. Of these isolates, only 19 % of the *S. anginosus* but almost all isolates of *S. intermedius* and most *S. constellatus* isolates were found to associate with purulent infection, in comparison to otherwise healthy subjects. This lower virulence potential reported for *S. anginosus* might be explained, in part, by the poor adherence/biofilm potential reported for *S. anginosus* in the above mentioned studies (Eick *et al*. 2004; Fernanda Petersen *et al*. 2004; Landrygan-Bakri *et al*. 2012; Takeshi Yamanaka 2013).

Alongside SAG, we tested the biofilm potential of *E. faecalis* which is frequently isolated from root canals of teeth following failure of endodontic procedures (Portenier *et al.* 2003; Ozbek

et al. 2009). Occasionally *E. faecalis* has been isolated from primary pulpitis (Ozbek *et al*. 2009). This means that this species is closely associated with the pathogenesis of endodontic infection and persistence of apical periodontitis. Herein, the results revealed the ability of this strain to grow and form consistent biofilm on polystyrene well plate. The number of viable bacterial cells recovered from *E. faecalis* biofilm was comparable to that of *S. intermedius*. This outcome is in accordance with the finding of Foley and Gilbert (1997), who demonstrated the growth of an immature *E. faecalis* biofilm after 12 h incubation that yielded variable cell counts whilst a mature consistent biofilm was obtained between 12 and 96 h incubation. Lima et al. (2001) showed that one day old biofilm contained more viable cells than 3 days-old one. Enterococcal adhesiveness and biofilm potential have been studied and a number of molecules identified as being involved in biofilm formation. For instance, aggregation substance (AS) is a surface adhesin that has been found to involve in the adhesion of *E. faecalis* to intestinal and renal epithelium and to components of the extracellular matrix (Süßmuth *et al*. 2000; Wells *et al*. 2000; Rozdzinski *et al*. 2001). Microbial surface components recognising adhesive matrix molecules (MSCRAMMs) have been documented to facilitate the colonisation of host tissues and binding of bacterial cells to indwelling abiotic surfaces that become coated with host-derived extracellular matrix components. *E. faecalis* has been examined for the presence of such molecules and shown to have more than a dozen MSCRAMMs which may be involved in adherence (Sillanpää *et al.* 2008). Moreover, an *E. faecalis* polysaccharide antigen locus (*epa*) has been labelled as being involved in the synthesis of cell wall-associated polysaccharides, which contribute to biofilm formation, among other virulence properties (Teng *et al.* 2002).

Adhesiveness and biofilm formation is an important characteristic required for probiotics to be used in the oral cavity so they can resist the normal oral transit. *L. plantarum* followed by *L. fermentum* were able to form the most robust consistent biofilm as measured by the recovery of viable cells grown on polystyrene well plate for 24, 48 and 72 h (Figure 2.6). There is no conclusive statement regarding the probiotic species biofilm potential. This might be due to the wide variety of probiotic strains tested, different experimental conditions for each study and different abiotic or cell-line surfaces. The differences in probiotic-binding capacity *in-vitro* may indicate that there are also differences in the persistence of the different probiotic strains in the oral cavity (Haukioja *et al*. 2006). The mechanism of *L. plantarum*

adherence has not been well documented. One study (Ramírez *et al*. 2015) demonstrated a high *L. plantarum* biofilm sensitivity to treatment with proteinase K suggesting a role of proteins and/or proteinaceous molecules in surface colonisation. Two further studies (Kubota *et al*. 2008; Samot *et al*. 2011) reported that *L. plantarum* produces certain polysaccharides which might contribute to its high adherence and good biofilm formation. *L. plantarum* strains were found to form biofilm with the highest cultivable cell count within 24 h; the cell count remained constant for 48 h but decreased after 72 h by 1 to 3 $\log_{10}$ per well (Ramírez *et al*. 2015). This finding is in accordance with the current study observations. In contrast, Jalilsood et al. (2015) reported that *L. plantarum* PA21 cell counts analysis (CFU/cm^2) revealed the highest biofilm cell density on the fourth day of culture.

The probiotic species that showed the least capacity to adhere and remain viable over the time course and was therefore regarded as the species with the lowest biofilm forming capacity was *S. thermophilus* NCTC 10353. This poor ability of *S. thermophilus* to grow in dense biofilm has been found, in one study, to be correlated to the poor initial adherence to the surface and to the lack of the activity of biofilm genes which are involved in surface polysaccharide production and exposed surface protein. This has been proposed to be due to the genetic domestication of *S. thermophilus* and adaptation to milk environment where it is used mostly (Couvigny *et al.* 2015).

It may be worth highlighting that in the microtiter plate biofilm assay method used herein, all adherent biomass was used to evaluate biofilm. However, bacterial cells that sedimented to the bottom surface of the wells and become embedded by the extracellular biofilm matrix might constitute part of the biofilm biomass. Therefore, the resultant biofilm biomass is not necessarily generated entirely as a result of biofilm formation (Azeredo *et al.* 2017).

Probiotics have been shown to play a beneficial role to the oral health via a number of proposed mechanisms. In this study, five probiotic strains; *L. plantarum*, *L. fermentum*, *L. acidophilus*, *L. lactis* and *S. thermophilus* were screened for their antimicrobial activity against SAG and *E. faecalis*. Extraction of new molecules from natural sources, such as probiotics, have proven to be a promising and interesting niche in the antimicrobial field, as most of these natural components may be difficult to synthesise and may prove safer than synthetic products (Kamat and Velho-Pereira 2012). As living microorganisms, probiotics produce several primary and secondary metabolites. Some of them may have antimicrobial activity,

which can be tested with different methods. Here, the antimicrobial activity of the probiotic strains was tested both during growth on solid agar in proximity to target species and in broth culture in co-culture of their supernatants with SAG and *E. faecalis*. Results demonstrated that all *Lactobacillus* treatments inhibited the growth of the tested bacteria but to a variable extent. *L. plantarum* demonstrated the most inhibitory activity against SAG members and *E. faecalis* followed by *L. fermentum*. At the time of writing this chapter, there have been no previous studies assessing the antimicrobial efficacy of this strain of *L. plantarum* against SAG/ *E. faecalis* as a Gram positive endodontic infection causative agent.

The cross streak assay was employed herein to screen for the antimicrobial potential of living probiotics cells. It has been shown previously that the cross streak assay resulted in higher inhibition zones on indicator bacteria than those obtained by the method of agar well diffusion (Lertcanawanichakul and Sawangnop 2011). It may be the case that some bacteria may only produce certain substances when growing on solid media, in comparison to the liquid state. For instance, the production of some bacteriocins from Gram positive bacteria has been found to occur preferentially in solid media, as in the case of the bacteriocin from *Actinomyces odontolyticus* (Franker *et al.* 1977). Pre-incubation was performed at different time periods from 1 to 10 days. This was done to provide enough time for the live organism to produce antimicrobial substances which might diffuse into the agar medium (Velho-Pereira and Kamat 2011).

L. plantarum showed the most inhibitory effect against SAG members and caused notable restriction of *E. faecalis* growth on full strength FAA plates. This antagonistic activity was prominent after prolonged (> 7 days) pre-incubation of the probiotic strains tested. *L. fermentum* also showed anti-SAG effect at prolonged pre-incubation. This may be attributed, in part, to the stress of nutrition limitation after this prolonged incubation period which caused the microorganism to produce inhibitory competitive molecules for their own survival This suggestion is supported by the finding of Tong et al. (2012). They observed an antagonism between *L. lactis* and *S. mutans* under *in-vitro* nutrition deficiency while this effect was lost when the two bacteria were grown in nutrition rich media. They proposed that, under nutrition limitation, quorum sensing system may be activated, which plays a key role in bacterial stress responses causing the bacteria to produce molecules, such as nisin, with inhibitory activity against other competing counterparts (Tong *et al*. 2012).

S. thermophilus NCTC 10353 did not demonstrate any inhibitory effects against any of the pathogenic strains tested in the current study, even after prolonged pre-incubation of 10 days. This may result from the unsuitability of FAA as a supporting medium for this species especially as it was noticed to grow poorly on FAA. However, Letort and Juillard (2001) stated that *S. thermophilus* appeared to be less nutrient-demanding than other lactic acid bacteria (Letort and Juillard 2001). This *S. thermophilus* NCTC 10353 strain may have no inhibitory effect against SAG and *E. faecalis*. Similarly, *S. thermophilus* M37 was tested for its antimicrobial activity against a number of pathogens, although no SAG or *E. faecalis* were included in the study, and no antimicrobial activity was demonstrated against any of the tested pathogens (Hladíková *et al.* 2012). Contrary to the current finding, *S. thermophilus* A has been shown to produce a glycolipid biosurfactant with antimicrobial/antiadhesive activity against several bacterial and yeast strains isolated from explanted voice prostheses (Rodrigues *et al*. 2006). Another study, conducted in 2011 on *S. thermophilus* strains isolated from a Turkish yogurt, demonstrated that all these strains showed inhibitive activity against at least one of the tested pathogens, although nutrient agar was used as a test medium (Akpinar *et al.* 2011).

A more selective/specific inhibitory effect of *L. lactis* NCTC 10353 was noticed herein. *L. lactis* inhibited *S. intermedius* and *S. constellatus* growth but only after prolonged pre-incubation of 9-10 days. This inhibitory activity was observed only when the bacteria grew on full-strength FAA plates. This may be due to the production of specific inhibitory substances which were synthesised in response to stress and might reflect as well the different antimicrobial susceptibility of the SAG members to different antimicrobial agents (Jacobs and Stobberingh 1996; Rams *et al.* 2014). In a study conducted in 2012, a selective antimicrobial activity was reported for two strains of *L. lactis* (ZS25 and LM25). *L. lactis* ZS25 inhibited the growth of *E. coli* (1.75 %) and *S. aureus* (5.25 %), while *L. lactis* LM25 inhibited the growth of *P. aeruginosa* (3.06 %) and *S. aureus* (3.75 %) among other tested pathogens (Hladíková *et al.* 2012). *L. acidophilus* did not show any inhibitory effect against any of SAG or *E. faecalis*. Contrary to the finding of current study, Chapman (2013) reported *L. acidophilus* as the most inhibitory amongst four probiotic strains against *E. faecalis* showing zone of inhibition of around 9 mm using agar spot assay (Chapman 2013). A study conducted in 2014 on *L. acidophilus* strains isolated form yogurt revealed antimicrobial activity against *B. subtilis*, *P. aeruginosa*, *S.*

pyogenes, *Proteus vulgaris*, *S. aureus* and *Aeromonas hydrophila* using the cell-free supernatant in a well diffusion method. PCR analysis revealed that these stains harbour the gene encoded for bacteriocin which might contribute to the observed inhibitory effect (Abdulla 2014). Moreover, antimicrobial activity was documented for three *L. acidophilus* strains against a number of foodborne pathogens. However, the diameter of inhibition zone varied between the three *L. acidophilus* strains against the different foodborne pathogens included in this study. This inhibitory activity was associated with the production of a bacteriocin-like substance (Abo-Amer 2013).

FAA agar was used in the cross streak assay because it supported the growth of both the fastidious probiotic (Liew *et al.* 2005) and the pathogenic strains. As bacterial production of certain molecules is correlated with the medium composition (Lin and Pan 2015) hence, FAA medium might have influenced, by enhancing or attenuating, the production of potential compounds from the probiotics. Two concentrations of FAA (full strength and half-strength) were used here as a try to mimic the situation in the progressed endodontic infection of deficient nutritional status so the activity of the bacteria (probiotics and pathogens) under this condition can be evaluated. *L. fermentum*, *L. acidophilus* and *L. lactis* but not *L. plantarum,* demonstrated a reduced antimicrobial activity when grow on half-strength FAA plates. This might indicate the requirement of specific elements at enough concentration for probiotics to synthesise their antimicrobial substance. Or it might be due to the increased resistance of the pathogenic strains of SAG and *E. faecalis* to the inhibitory effect of the probiotics when grow on deprived medium.

To measure the inhibitory effect of the probiotic cell-free supernatant on planktonic growth of the pathogens, a broth inhibition assay was conducted. Results indicated that cell-free supernatant of certain probiotics reduced the growth of certain pathogens, when there was no adjustment of pH (acidic pH). This inhibitory effect might be attributed to the pH-lowering potential of acid production. This is emphasised by the disappearance of some of the observed antimicrobial effect upon neutralisation of the pH (by addition of 1 M NaOH) to pH 7.0). It was proposed that one of the probiotic antimicrobial mechanisms is acid production (O'Hanlon *et al.* 2011) as a result of fermentation process such as lactic and acetic acid (Alakomi *et al*. 2000; De Keersmaecker *et al*. 2006; Makras *et al*. 2006). Lactic acid has been shown to inhibit the growth of many pathogens including species of

Enterobacteriaceae and *Pseudomonadaceae* (Davidson *et al.* 2005). Lactic acid can penetrate the bacterial cytoplasmic membrane and cause a reduction in the internal pH. This, in turn, leads to the disruption of the transmembrane proton motive force (Ray 1992). Alakomi and colleagues (Alakomi *et al.* 2000) proposed an additional (or alternate) mechanism of lactic acid effect, which was its ability to disintegrate the outer membrane of Gram negative bacteria at low pH (3-4). This permeabilisation has been proved by the release of lipopolysaccharide (LPS) and sensitisation of the bacteria to detergent or lysozyme effects. Hence, lactic acid, in addition to its antimicrobial effect by the low pH, can work as adjuvant to enhance the bacterial penetration of other antimicrobials.

The acid-related antimicrobial activity noticed in the current study was emphasised by the partial abrogation of the observed effect upon neutralisation of the pH as mentioned earlier. This was documented particularly against *E. faecalis*; its significant growth suppression by the crude lactobacilli supernatants was absent when the pH was adjusted to 7 (Figure 2.9a). This result is in agreement with the finding of a previous research study (Chapman *et al.* 2013) that assessed the crude supernatant effect of four probiotic lactobacilli (*L. acidophilus* NCIMB 30184 (PXN 35), *L. fermentum* NCIMB 30226 (PXN 44), *L. plantarum* NCIMB 30187 (PXN 47) and *L. rhamnosus* NCIMB 30188 (PXN 54) against two urinary tract pathogens (*E. coli* NCTC 9001 and *E. faecalis* NCTC 00775). The crude supernatants of the lactobacilli (except for *L. rhamnosus*) significantly reduced the growth of *E. faecalis* (measured by absorbance at 650 nm), nonetheless this effect was lost when pH adjusted to 7 indicating an acid-induced inhibitory activity (Chapman *et al.* 2013).

Interestingly, the current study demonstrated that not all neutralised supernatants lost their growth antagonist effect upon pH neutralisation, as in the case of *L. plantarum, L. fermentum* and *L. acidophilus*, which may be attributed to the production of other compounds with antibacterial activity. These results are supported by the previous study of Terai et al. (2015) who screened a number of probiotics for their potential against a number of periodontal and cariogenic oral pathogens. Antimicrobial activity of 3 lactobacilli cell culture supernatants against two pathogens (*Porphyromonas gingivalis* ATCC 33277, *Aggregatibacter actinomycetemcomitans* ATCC 43718) was reported even after pH adjustment (Terai *et al.* 2015). The different organic acids of lactic acid bacteria, in general, have been reported to contribute to about 50 % of the reported antimicrobial effect (Olivares *et al.* 2006). The

remaining antagonistic effects may be due to the synthesis and excretion of other antimicrobial compounds such as hydrogen peroxide, bacteriocins and/or biosurfactants (Piard and Desmazeaud 1991; Piard and Desmazeaud 1992; Delgado *et al.* 2001; Madhu and Prapulla 2014). According to Piard and Desmazeaud (1991) the production levels and the proportions among these compounds depend largely on the strain, medium composition and other physical parameters.

The probiotic supernatants tested in this chapter were filter-sterilised. This excludes the possible probiotic-pathogen bacterial cells competition for nutrients which is a potential mechanism of antimicrobial effect in the agar streak assay. Instead, it could be that during their overnight incubation, the probiotics produced bacteriocins, a small antimicrobial peptides which can act towards other bacterial species with greater potency via such mechanisms as pore formation and inhibition of cell wall synthesis (Coman *et al.* 2014).

Although *S. thermophilus* is considered a lactic acid bacterium, its cell-free supernatant was found to be only slightly acidic (pH = 6.5 ± 0.1). A study has demonstrated that the levels of lactic acid production by *S. thermophilus* isolates from traditional yogurt varied depending on the strain and the growth conditions used. By using HPLC, lactic acid production was measured and found to range between 0 and 77.9 mg/kg for the different *S. thermophilus* strains studied, which suggested a strain specific property (Gezginc *et al.* 2015).

Collectively, the inhibitory activity of *L. plantarum* both in cross streak assay and broth inhibition assay strongly suggests the ability of this bacterium to produce inhibitory substances in solid and liquid media. The differences in the results between cross streak and broth inhibition may be due in part to the different testing protocols. In the cross-streak assay, viable cells were used and incubated for long period of time until the significant inhibitory effects were noticed which may be due to factors other than acid production such as competition for nutrients and production of other antimicrobial compounds. However, the antimicrobial activity in liquid media is favoured by rapidly diffusing antimicrobial compounds such as organic acids and bacteriocins (Coman *et al.* 2014). The broth inhibition assay is based on using cell free supernatant of 24 h probiotic broth which may contain higher concentrations of acids than that produced by the cells growing on solid media.

2.5 Conclusion

Of the species tested, *L. plantarum* showed the broadest antimicrobial effect both in solid and liquid media together with the highest biofilm forming potential and therefore was considered to be a good candidate for further investigation. *L. fermentum* also revealed a biofilm formation potential and inhibitory effects comparable to that of *L. plantarum,* but mainly in full-strength medium. SAG and *E. faecalis* showed differences in response to probiotic treatment with the *E. faecalis* being less susceptible. *E. faecalis was* one of the more successful biofilm forming species, in addition to S*. intermedius*.

3 Chapter 3 *L. plantarum* biosurfactant extraction, characterisation and purification

3.1 Introduction

Probiotic agents including lactobacilli have been studied extensively for their beneficial health properties (Santos *et al.* 2009; Bermudez-Brito *et al.* 2012) including an ability to interfere with pathogenic infections via a variety of mechanisms (see Chapter 1, General introduction). Production of biosurfactants by probiotics is found to be one of these interfering mechanisms (Rodrigues *et al.* 2006; Sharma and Saharan 2016; Morais *et al.* 2017). Biosurfactants are a structurally diverse group of amphiphilic compounds with both hydrophilic and hydrophobic moieties (mostly hydrocarbons) which can affect surfaces, lower surface and interfacial tensions, form micelles, and exert emulsifying effects on immiscible materials (Chen *et al.* 2007; Franzetti *et al.* 2010; Pacwa-Płociniczak *et al.* 2011; Souza *et al.* 2014; Varjani and Upasani 2016). Biosurfactants have been under extensive investigation to be used as replacements for synthetic surfactants and have shown promise in a variety of industrial and environmental applications (Varjani and Upasani 2017). Chemically, biosurfactants can be grouped either as low or high molecular weight; the former consisting of glycolipids and lipopeptides and the latter of high molecular weight polymeric biosurfactants (Yu and Huang 2011; Hosková *et al.* 2013; Ramírez *et al.* 2015). Microorganisms can synthesise biosurfactants as nonpolar hydrocarbons, as well as in water-miscible medium, and the produced biosurfactants have been shown to be released into the growth medium or remain attached to the cell wall (Mukherjee *et al.* 2006). Lactobacilli have been documented in a number of studies as "biosurfactant producers" (Gudiña *et al.* 2011; Satpute *et al.* 2016; Morais *et al.* 2017). However their biosurfactants are found to be less effective in reducing surface tension (reducing surface tension of water to 36-40 mN/m) and are synthesised in lower biomasses (20-100 mg/L). Despite the increasing interest in *Lactobacillus* biosurfactants, there is minimal information available about their chemical composition and this impact on their commercial production. Only few studies have examined the *Lactobacillus* biosurfactants in depth, characterising them partially as multipotent complex of proteins, polysaccharides and/or lipids (Velraeds *et al.* 1996; Velraeds *et al.* 1996; Busscher *et al.* 1997; Gudina *et al.* 2010; Shokouhfard *et al.* 2015). A wide range of analytical, extraction, purification and characterisation methods, including precipitation, filtration, solvent extraction,

centrifugation, foam fractionation and chromatographic techniques have been employed for biosurfactants production (Heyd *et al.* 2008; Pornsunthorntawee *et al.* 2008; Banat *et al.* 2010; Varjani and Upasani 2016). In addition to these techniques, more sophisticated methods such as ion exchange, adsorption and desorption can be used for biosurfactants purification (Satpute *et al.* 2010; Varjani and Upasani 2017). To characterise the extracted biosurfactants, a number of techniques including thin layer chromatography (TLC), gas chromatography (GC), fourier transform infrared spectroscopy (FT-IR), gas chromatography–mass spectrometry (GC–MS), electrospray ionisation mass spectrometry (ESI-MS), high-performance thin layer chromatography (HP-TLC), high performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC–MS), fast atom bombardment–mass spectrometry (FAB-MS) and nuclear magnetic resonance (NMR) have been used (Benincasa *et al.* 2004; Abdel-Mawgoud *et al.* 2009; Raza *et al.* 2009; Lotfabad *et al.* 2010; Satpute *et al.* 2010; HoŠková *et al.* 2013; Varjani and Upasani 2016). Light scattering and microscopic hydrodynamic protocols have been used to obtain micelles characteristics (Satpute *et al.* 2010).

As previously shown in Chapter 2, *L. plantarum* demonstrated the broadest antimicrobial activity against the four endodontic isolates and was chosen as the potential probiotic for biosurfactants production and extraction. To our knowledge, no other studies have assessed the antimicrobial/antiadhesive activity of *L. plantarum* biosurfactants against SAG and *E. faecalis*. The aim of this chapter was to screen for the best growth phase of *L. plantarum* strain for biosurfactant production and to determine the biosurfactant production profile. Biosurfactant physical characterisation including surface tension and emulsifying activity determination was also undertaken. To provide an insight into the chemical composition of the extracted biosurfactants, partial chemical characterisation has been also performed.

3.2 Materials and methods

3.2.1 Biosurfactant extraction

3.2.1.1 Growth of *L. plantarum* for biosurfactant production and extraction

Biosurfactant production by *L. plantarum* was conducted in 500 mL flasks containing 400 mL of MRS medium. Each flask was inoculated with a pre-culture grown overnight at 37 °C and in 5 % CO_2 and adjusted to an OD_{600} of 0.08-0.1 (10^8 CFU/mL) and diluted 1:10 to get a bacterial suspension of around $1x10^7$ CFU/mL. The later was then incubated at 37 °C and 5 % CO_2. To determine the best time for biosurfactant production, four time points during the standard growth curve of *L. plantarum* (see Section 2.2.5) were selected which represented the mid-exponential phase (8.5 h), the early stationary phase (16 h) and two points of the late stationary phase at 24 h and 48 h.

At the end of each time point, the 400 mL sample was collected and ultracentrifuged at 10,000 *g* at 10 °C for 10 min (SORVALL RC +6 centrifuge Thermo Scientific USA). The bacterial pellet was used for cell-bound PBS-extract recovery (see Section 3.2.1.2). The supernatant (MRS-extract) was collected and filtered through 0.22 µm filter unit (Whatman, GE Healthcare, UK) and used for surface tension measurements looking at the released biosurfactant (see Section 3.2.1.3).

3.2.1.2 Cell-bound PBS-extract recovery

The bacterial pellet was washed twice with the 400 mL of sterile double distilled water, and resuspended in the same volume of phosphate-buffered saline (PBS: 10 mM KH2PO4/K2HPO4, pH adjusted to 7.0) (Vecino *et al.* 2015). The bacterial-suspension was left at room temperature (20-25 °C) for 18 h with gentle stirring for cell-bound biosurfactants release. Subsequently, bacteria were removed by centrifugation (10000 *g*, 10 °C for 10 min) and the remaining supernatant was filtered through a 0.22 µm filter unit (Whatman, GE Healthcare, UK). To confirm biosurfactant production, the surface tension was measured (Section 3.2.1.3) during the extraction process at each time point.

3.2.1.3 Surface-activity determination

The surface tension of the bacterial supernatants (MRS-extract) and the cell bound PBS-extracts were evaluated using the Du-Nouy-Ring method based on measuring the force required to detach a ring or loop of wire from an interface or surface (Tadros 2005). Dynamic

contact angle analyser (KR USS GmbH, Germany) equipped with a 1.9 cm De Nouy platinum ring (Thermal exchange Ltd, Germany) was used. The machine was equilibrated with distilled water twice before use. Sterile PBS was used as a negative control for cell-bound PBS-extract. Sterile MRS medium was used as the negative control for the broth extract. All the measurements were performed at room temperature (20-25 °C) in triplicates.

3.2.1.4 Biomass determination

For biomass determination, 400 mL samples of the MRS-extract or the cell-bound PBS-extract were freeze-dried and the resulted powder was weighed. The yield was expressed as milligram of the sample per millilitre of the culturing medium (mg/mL).

3.2.1.5 Dialysis

To remove PBS, the freeze-dry cell-bound PBS-extract product collected at the mid-exponential phase was dissolved in 5 mL of sterile distilled water and then dialysed against distilled water at 4 °C using dialysis membrane of molecular weight cut-off 1000 Da (Spectra/Por 6 Membranes; Cole-Parmer instrument Ltd. UK) for 48 h with three changes of the dialysis solution. The dialysate was further filter sterilised and freeze-dried. The crude dialysed biosurfactant (termed *Lp*-BS from here forth) was weighed and stored at -20 °C for subsequent studies.

3.2.2 Physicochemical characterisation of *Lp*-BS

3.2.2.1 Physical characterisation: Surface tension and emulsifying activity determination

Surface tension was determined as discussed in section 3.2.1.3. Emulsifying activity was determined by the addition of 2 mL of either kerosene (Sigma Aldrich), heptane (Sigma Aldrich), hexane (Sigma Aldrich), xylene (Fisher Scientific), motor oil or sunflower oil to the same volume of distilled water and 1 mL of *Lp*-BS solution (at a concentration of 1 mg/mL in distilled water) in glass test tubes. The mixtures were mixed by vortexing at high speed for 2 min and subsequently incubated at 25 °C for 24 h. The emulsification indexes (EI24) were calculated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm) as follows:

$$EI24\ (\%) = \alpha / \beta \times 100$$

, where α and β represent the height of the emulsified layer and the total height, respectively. All measurements to determine the emulsification indexes were performed in triplicate.

3.2.2.2 Biochemical characterisation of *Lp*-BS

3.2.2.2.1 Fourier transform infrared spectroscopy (FT-IR)

Fourier transform infrared spectroscopy using Nicolet 380 FT-IR (Fisher Scientific) was used to study the molecular composition of *Lp*-BS. The spectra were recorded on FT-IR-4100 Spectrometer and were obtained from 64 scans with a resolution of 8 cm in the range of 600–4000 cm^{-1}. Samples were prepared via homogeneous dispersal of 30 mg of *Lp*-BS sample in pellets of potassium bromide (KBr) powder at a ratio of 1:100 and pressed under 7,500 kg of pressure to gain translucent pellet. IR spectra were collected over the 600 and 4000 cm^{-1} range. A pellet of KBr was used as a background reference.

3.2.2.2.2 Lipid detection by thin layer chromatography (TLC)

To detect for lipid presence in the *Lp*-BS sample, 100 µL aliquot of *Lp*-BS (prepared at concentration of 1 mg/mL in distilled water) was spotted using a pipette on a precoated 5cm×10cm silica gel plate (Sigma Aldrich). Alongside the sample, rhamnolipid (1 mg/mL) (Sigma Aldrich) was also spotted onto the plate as a positive control. The mobile phase used for the separation of the biosurfactants composed of chloroform (Fisher Scientific, UK)/ methanol (Fisher Scientific, UK)/acetic acid (Fisher Scientific, UK) at a ratio of 65:15:2 v/v respectively. The developing jar was allowed to be saturated with the mobile solvent system for 20-30 min. The spotted plate was then place in the jar and monitored by eye until the mobile phase reached near the top end of the plate. After the separation of lipid mobile components, the plate was then sprayed with the detection agent consisting of ammonium molybdate/cerium sulphate (0.42 % w/v ammonium molybdate and 0.2 % w/v cerium (IV) sulphate in 6.2 % v/v sulphuric acid). The plate was then heated in the oven at 120 °C for 10 min. The chromatogram of *Lp*-BS was compared with the TLC pattern of a standard mixture of rhamnolipids (mono and di-rhamnolipid). Brown staining bands indicated the presence of lipid in the tested solutions.

3.2.2.2.3 Protein assay, SDS-PAGE and silver staining

3.2.2.2.3.1 Bicinchoninic Acid Protein Assay (BCA)

To determine total protein concentration in the bacterial extracts (PBS-extracts and MRS-extracts) obtained at the four time points selected, a Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, USA) was used. This assay involved the addition of BCA reagent (composed of bicinchoninic acid and cupric sulphate) to the protein-containing solution. Copper ions in the cupric sulphate are reduced by the peptide bonds in the protein molecules. The reduced ions are then chelated by the bicinchoninic acid which causes the formation of a purple product which can be measured colorimetrically. The more protein in the sample, the more ions are reduced and this is proportional to the colour change and the absorbance reading.

A standard curve was generated according to the manufacturer's instructions, by the addition of 25 µL of a range of concentrations from 25 µg/mL to 2,000 µg/mL of known bovine serum albumin (BSA) standards to the corresponding wells of a 96 well plate (SARSTEDT, Germany) in triplicate. On the same plate in triplicate, 25 µL of the PBS-extracts or MRS-extracts (1 mg/mL in distilled water) for the four time points selected for the extraction of the biosurfactant were included. Sterile MRS or PBS were also placed into triplicate wells as control. Two hundred microliters of BCA reagent [reagent A: bicinchoninic acid in 0.1M sodium hydroxide and reagent B: 4 % (w/v) cupric sulphate, (50:1, reagent A: B)] was then added to each of the standards and samples. The plate was shaken for 30 s using a plate shaker and then incubated at 37 °C for 30 min. At the end of incubation period, the plate was left to cool down to ambient temperature. Absorbance was read at 562 nm using a SPECTROstar Omega Plate Reader (BMG Labtech, Germany). By plotting the absorbance values from the BSA standards vs. the concentrations, a standard curve was generated and a best-fit equation line was obtained with Microsoft® Excel®2013 which allowed the calculation of the test samples concentrations.

3.2.2.2.3.2 Gel electrophoresis using SDS-PAGE and sliver staining

Double concentration (2x) Laemmli Sample Buffer (BioRad Laboratories, USA) was supplemented with 2-mercaptoethanol (Sigma-Aldrich) at 5 % (v/v) and diluted 1:1 with distilled water to generate 1x Laemmli Sample Buffer. Twenty µg of protein samples (determined previously in Section 3.2.2.2.3.1) was diluted 1:1 with 1x Laemmli buffer (Bio-Rad, USA). The resultant mixture was heated to 95 °C for 5 min and 18 µL were loaded on 4-

15 % Mini- Protean® TGX™ Precast Gels (Bio-Rad) along with 8 µL Kaleidoscope™ Pre-stained Standard Markers (Bio-Rad). Gels were placed into a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (BioRad Laboratories, UK) in 1x sodium dodecyl sulphate-polyacrylamide gel-electrophoresis (SDS-PAGE) running buffer [1.92 M glycine (Thermo Fisher Scientific), 250 mM tris-base (Sigma-Aldrich), 1% SDS, pH 8.3). Separation of proteins was performed by electrophoresis at 200 V/cm^2 for 35-40 min, until the migration front reached the bottom of the gel.

To visualise the separated protein bands in the gel, silver staining was performed using a Silver Stain Plus™ kit (BioRad Laboratories). The SDS-PAGE gel was removed from the casts and, according to the manufacturer's protocol, placed in the fixative solution [(50 % v/v methanol (Fisher Scientific), 10 % v/v acetic acid (Fisher Scientific), 1% v/v Fixative Enhancer Concentrate, 30 % v/v distilled water)] with gentle agitation for 20 min. A 10 min wash of the fixed gel was performed twice with 400 mL double distilled water. Staining solution was prepared fresh by adding 5 mL Silver Complex Solution, 5 mL Reduction Moderator Solution and 5 mL Image Development Reagent to 35 mL double distilled water, with gentle stirring followed by the addition of 50 mL Development Accelerator Solution to the staining solution. The washed gel was then placed in the staining solution for approximately 20 min with gentle agitation until the required staining intensity was obtained. Immediately after, the staining reaction was stopped by decanting the staining solution and adding 5 % v/v acetic acid for 15 min with a final wash in double distilled water for 5 min. The gel was imaged using a Gel Doc EZ System (BioRad Laboratories).

3.2.2.2.4 Quantitative assessment of carbohydrate content by anthrone assay

To quantify carbohydrate content in *Lp*-BS, an anthrone test was performed. Anthrone reagent was prepared by dissolving 0.2 g of crystalloid anthrone (Fisher Scientific, UK) in 71 mL concentrated > 99.7 % sulphuric acid (Fisher Scientific, UK) in an ice bath. After complete dissolution, the acid solution was added to 29 mL cold double distilled water with gentle agitation allowing minimal heat generation. A range of glucose standard concentrations (10-50 µg/mL) were prepared. Two and a half mL of anthrone reagent was added to each of the test or standard samples in glass tubes in an ice bath. A 0.5 mL volume of the crude biosurfactant solution at a concentration of 1 mg/mL in double distilled water, the standards and the water blank was layered on top of the anthrone reagent tubes. The tubes were then

heated at 95 °C in a water bath for 10 min. The formation of a bluish green coloured complex indicated the presence of carbohydrate and the absorbance at 620 nm was measured. Standard equation was generated from the best fit line and total carbohydrate was drawn using glucose as substrate.

3.2.3 Fractionation by size exclusion chromatography

For purified fractionation of the biosurfactant components, a Sephacryl gel filtration column (HiPrep™ 16/60 Sephacryl® S-100 HR, Sigma Aldrich) of fractionation range 1KDa-100KDa, was used. *Lp*-BS was dissolved in phosphate buffer (0.01 M sodium phosphate buffer, 0.15 M NaCl, pH 7.0) at a concentration of 4 mg/mL and filtered through a 0.22 µm filter unit to remove of any insoluble matter. Size exclusion chromatography was performed, using AKTA Purifier apparatus controlled by Unicorn Software (GE Healthcare, v5.2). The system, into which was connected the Sephacryl® S-100 HR column, was washed in 20 % (v/v) ethanol and pumped with 18.2 MΩ water and then equilibrated with 0.01 M sodium phosphate buffer, 0.15 M NaCl (pH 7.0). Two mL of *Lp*-BS solution was loaded into the column and eluted out with phosphate buffer at a flow rate of 30 mL/h. The absorbance of the eluent from the column was continually monitored at 210 nm, 254 nm and 280 nm. Eluent from the column was collected in 5 mL fractions and subsequently pooled into five elution pools labelled as F1, F2, F3, F4 and F5. The collected five elution pools were transferred to dialysis tubing (Spectra/Por 6 Membranes; Cole-Parmer instrument Ltd. UK, MWCO 1000Da) and dialysed against double distilled water for 48 h at 4 °C, with continuous agitation and with the dialysis solution being changed thrice daily. Dialysed fraction pools were frozen at -80°C and lyophilised for subsequent analysis.

3.2.3.1 Characterisation of elution pools fractionated by chromatography

3.2.3.1.1 Detection of carbohydrate

To determine carbohydrate presence of the five fractions, anthrone assay was employed as described previously in section 3.2.2.2.4.

3.2.3.1.2 SDS-PAGE and colloidal coomassie blue stain of proteins in the FPLC fractions

One milligram of each elution pools was dissolved in 1 mL distilled water and was electrophoretically separated by SDS-PAGE as described previously (Section 3.2.2.2.3.2). The gel was then stained with colloidal coomassie stain according to the manufacture's instruction

(Colloidal Blue Staining Kit, Thermo Fisher Scientific). The gel was gently shaken in the fixing solution [(40 mL distilled water, 50 mL methanol 99.9 % v/v (Fisher Scientific, UK), 10 mL acetic acid 99.9 % v/v (Fisher Scientific, UK)] for 10 min at room temperature. The gel was then transferred into the staining solution (55 mL distilled water, 20 mL methanol 99.9 % v/v (Fisher Scientific, UK), 20 mL stainer A) for 10 min at room temperature. Five mL stainer B were then added to the existing staining solution and the gel was shaken gently in the latter mixture for a minimum of 3 h. The staining solution was decanted and the gel was washed in 200 mL distilled water overnight at room temperature. For storage until analysis, the gel was kept in 1% v/v acetic acid at 4 °C.

3.2.3.1.3 Assessment of the emulsifying activity

Fraction 1 and 2 were analysed for emulsifying activity by employing emulsification index 24 assay against xylene (described in Section 3.2.2.1).

3.2.4 Mass spectrometric analysis of *Lp*-BS

In-gel proteomic analysis was performed at the Proteomics Facility, University of Bristol. Fifty µg of *Lp*-BS was electrophoretically separated by SDS-PAGE and the gel was stained with colloidal coomassie stain as described in section 3.2.3.1.2 and kept hydrated in distilled water whilst transported to Bristol Proteomic Facility centre. Ten major bands were selected (most intense ones), cut and placed in labelled microcentrifuge tubes. Each gel slice was subjected to in-gel tryptic digestion using a DigestPro automated digestion unit (Intavis Ltd.). The resulting peptides were dried down in a speedvac and then resuspended in 1% formic acid and fractionated using an Ultimate 3000 nano-LC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). In brief, peptides in 1 % (v/v) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific) which binds all peptides. After washing the nano-trap column with 0.5 % (v/v) acetonitrile 0.1 % (v/v) formic acid to remove any residual salts, peptides were loaded on a 250 mm × 75 µm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific). Then, an organic gradient was applied over a 60 min starting with 1 % solvent B/ 99 % solvent A. Over the next 39 min the % of solvent B increased (and the % of solvent A decreased) until a 50 % B/50 % A was reached, then % of solvent B was stepped up to 90 % (and the % of solvent A was dropped to 10 %) over the next 0.5 min and remained at this level for the next 4.5 min. After that, % of solvent B was dropped

back to 1 % (and % of solvent A was increased to 99%) with a flow rate of 300 nL/min. Solvent A was 0.1 % formic acid and Solvent B was aqueous 80 % acetonitrile in 0.1 % formic acid. Peptides were ionised by nano-electrospray ionisation at 2.1 kV using a stainless-steel emitter with an internal diameter of 30 µm (Thermo Scientific) and a capillary temperature of 250 °C. Tandem mass spectra were acquired using an LTQ- Orbitrap Velos mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific) and operated in data-dependent acquisition mode. The Orbitrap was set to analyse the survey scans at 60,000 resolution (at m/z 400) in the mass range m/z 300 to 2000 and the top twenty multiply charged ions in each duty cycle selected for MS/MS in the LTQ linear ion trap. Charge state filtering, where unassigned precursor ions were not selected for fragmentation, and dynamic exclusion (repeat count, 1; repeat duration, 30s; exclusion list size, 500) were used. Fragmentation conditions in the LTQ were as follows: normalised collision energy, 40 %; activation q, 0.25; activation time 10ms; and minimum ion selection intensity, 500 counts.

The raw data files were processed and quantified using Proteome Discoverer software v1.4 (Thermo Scientific) and searched against the UniProt *Lactobacillus plantarum* database (28578 sequences) using the SEQUEST algorithm. Peptide precursor mass tolerance was set at 10ppm, and MS/MS tolerance was set at 0.8Da. Search criteria included carbamidomethylation of cysteine (+57.0214) as a fixed modification and oxidation of methionine (+15.9949) as a variable modification. Searches were performed with full tryptic digestion and a maximum of 1 missed cleavage was allowed. The reverse database search option was enabled and all peptide data was filtered to satisfy false discovery rate (FDR) of 1 %.

3.2.5 Statistical analysis

Results were represented as mean ± standard deviation (SD) of three independent experiments. No statistical analysis was required in this chapter.

3.3 Results

3.3.1 Biosurfactant production

A growth curve was produced for *L. plantarum* and four time points for sample collection were selected in order to investigate biosurfactant production (Figure 3.1). The reduction in surface tension of the culture broth for samples collected at the four time points was minimal (2 mN/m - 0mN/m) (Table 3.1). This finding indicates that culture supernatant is not a good source of biosurfactant at any point in the growth phase. However, biosurfactant activity was detected from the cell pellet based PBS extraction procedure; over a period of 15 h at room temperature with gentle stirring, cell bound biosurfactant appeared to be released into the PBS medium.

Cell-bound PBS extract samples collected at the mid (8.5 h), early (16 h) and the late stationary phase (24 and 48 h) exhibited a reduction in surface tension at varying degree (17.3 ± 0.5, 11.8 ± 1, 16.6 ± 0.6 and 9.3 ± 0.6 mN/m respectively) when compared to PBS as a negative control. Although the 16 h-extract was found to produce the highest yield of the cell-bound biosurfactant (1.19 ± 0.3 mg per mL culture broth), the maximum surface tension lowering effect was observed for the mid-exponential phase sample together with a high yield of 1.0 ± 0.1 mg/mL (Table 3.1). In view of these results, the mid-exponential time point was selected for ongoing biosurfactant extraction and characterisation and is referred to as *Lp*-BS henceforward. Upon dialysis, the biomass of the obtained *Lp*-BS was reduced to 40 mg per litre of culture medium.

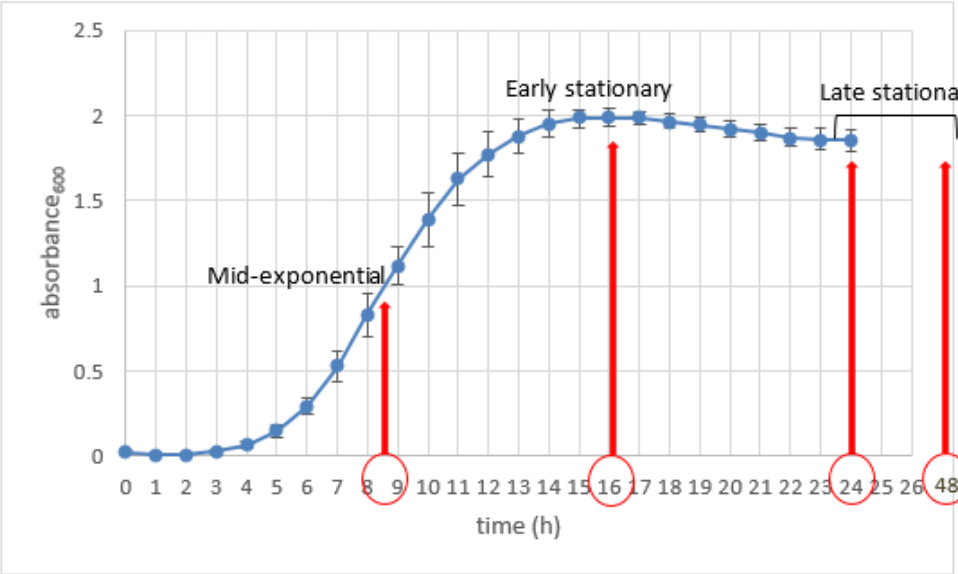


Figure 3.1 ***L. plantarum* growth curve.** Red arrows represent the four time points for sample collection and subsequent biosurfactant extraction. These time points represent the different phases of *L. plantarum* growth.

Table 3.1 **Comparison of the surface activity and yield of the MRS-extract and PBS-extract among different time points of *L. plantarum* growth.** Surface tension measured by means of Du Nouy ring. PBS-extract samples collected at the four time points reduced the surface tension of PBS. Negligible surface activity was monitored for the MRS-extract collected at any time. Data expressed as the mean ± SD of three separate experiments. The downward arrows represent the reduction in surface tension compared to the corresponding control.

	Cell-bound PBS-extract		**Extra-cellular MRS-extract**	
Time of sample collection	Yield (mg/mL)	ST (mN/m)	Yield (mg/mL)	ST (mN/m)
Mid-exponential (8.5 h incubation)	1.00 ± 0.10	↓17.3 ± 0.50	15.0 ± 0.30	↓2.0 ± 0.03
Early stationary (16 h incubation)	1.19 ± 0.30	↓11.8 ± 1.00	15.0 ± 0.15	↓0.0 ± 0.00
Late stationary (24 h incubation)	0.60 ± 0.15	↓16.6 ± 0.60	15.0 ± 0.10	↓1.0 ± 0.50
Late stationary (48 h incubation)	0.80 ± 0.05	↓9.30 ± 0.60	16.0 ± 1.10	↓0.0 ± 0.00
Rhamnolipid was used as a positive control. It lowered surface tension of distilled water by 40 mN/m. MRS and PBS were used as negative controls with ST of 44 mN/m and 72 mN/m respectively.				

3.3.2 Emulsification activity

The surface activity of the *Lp*-BS was additionally assayed for emulsification effect against four hydrocarbon compounds and one vegetable oil and motor oil (Figure 3.2). Comparative analysis demonstrated that *Lp*-BS and rhamnolipid were able to emulsify kerosene, heptane and xylene to the same extent (EI24 % = 28.5 %) (Figure 3.3). A higher emulsifying index of heptane was recorded when mixed with *Lp*-BS or rhamnolipid than the synthetic surfactant tween80 (EI24 % = 28.5 % vs. 14.5 % respectively) (Figure 3.3). Tween80 was shown able to completely emulsify xylene (EI24 % = 100 %) while *Lp*-BS and rhamnolipid had an emulsifying activity of only 28.5 %. Rhamnolipid, on the other hand, showed a higher emulsifying activity (EI24% = 57.14%) against hexane than tween80 (EI24 % = 28.5 %) while *Lp*-BS failed to emulsify this hydrocarbon (Figure 3.3). The vegetable oil tested was shown to be emulsified by rhamnolipid and tween80 to the same extent (EI24 % = 28.5 %) while only the latter was shown to emulsify motor oil (EI24 % = 28.5 %). The control (distilled water) had an expected activity close to zero against the assayed hydrocarbons (Figure 3.3).

Figure 3.2 **Emulsification index 24 (EI24) of *Lp*-BS**. Six different hydrocarbons were used. Rhamnolipid was used as biosurfactant control and tween80 was used as a synthetic surfactant control. Distilled water (DW) employed as a negative control. EI24 (%) = α / β ×100, where α and β represent the height of the emulsified layer and the total height, respectively as represented in the figure. The figure is a representative of three independent repeats.

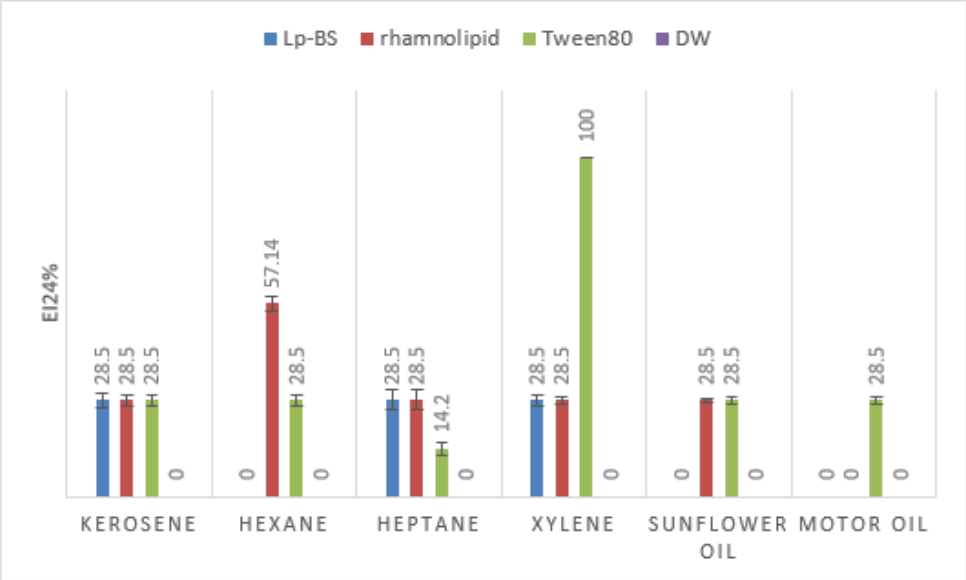


Figure 3.3 **Comparative emulsification activity of the cell-bound BS** on different hydrocarbons (kerosene, hexane, heptane and xylene), sun flower and motor oil. Rhamnolipid was used as a positive control for the biosurfactant and tween80 for the synthetic surfactant. The extracted *Lp*-BS showed rhamnolipid-comparable activity on kerosene, heptane and xylene. The error bars represent the mean ± SD (n=3).

3.3.3 Partial chemical characterisation

3.3.3.1 Fourier transform infrared spectroscopy (FT-IR)

To acquire a preliminary characterisation of the biosurfactant mixture, fourier transform infrared spectroscopy (FT-IR) was used to characterise the molecular composition of the cell-bound biosurfactant of *L. plantarum* (*Lp*-BS). The IR spectrum can be divided into two main regions, the functional group region and the fingerprint region. The former lays between 4000 cm^{-1} and 1000 cm^{-1} and it is where most of the functional groups show absorption bands. The right-hand third fingerprint region (about 1000 cm^{-1} -400 cm^{-1}) is characteristic of the compound as a whole and each compound shows a unique pattern in this region even if it shares a similar absorption bands in the functional region with other compound. Different functional groups were identified here and the most relevant peaks obtained are identified in figure 3.4.

The broad peak at 3350 cm^{-1} indicated the presence of OH and NH groups, characteristic of proteins. The strong and well defined peak at 1660.5 cm^{-1} corresponded to C=O stretching

(AMI protein band). The other peak at 1530 cm^{-1} was typical of N-H bending (AMII protein band). The identification of these groups suggests the presence of proteins in the examined sample. C-H stretching was detected at the small peak of the transmittance range 2948 cm^{-1} and the very small peak at 1461 cm^{-1}, corresponding to C-H bending (scissor) indicated the presence of bonds of aliphatic chains. The large well defined peak demonstrated at 1062 cm^{-1} was equivalent to the PII polysaccharide band typical of bond vibrations in the C-O-C group. Furthermore, another small but well defined peak was detected at 1232c m^{-1} which was a corresponding to C-O stretching in sugars.

A similar FT-IR spectrum (Figure 3.5) was evident for cell-bound biosurfactant of *L. agilis* (Gudiña *et al.* 2015) and was proposed by the authors to be a glycoprotein. The spectra of *Lp*-BS and *L. agilis* cell-bound biosurfactant had similar functional group region but different fingerprint regions.

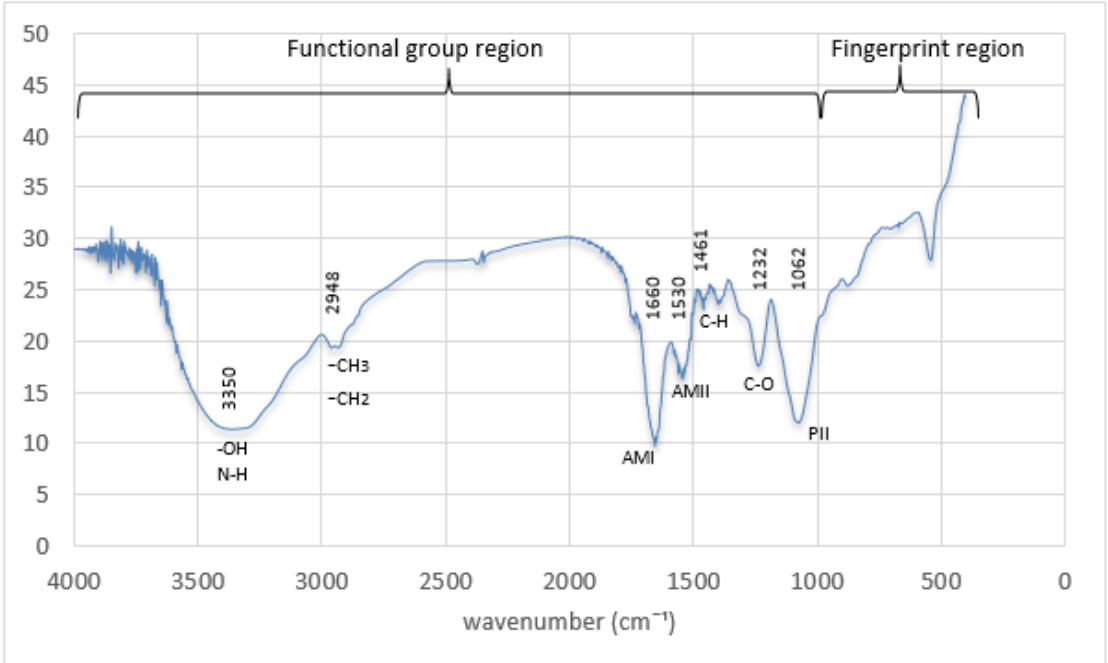


Figure 3.4 **FT-IR spectrum of *Lp*-BS**. Fourier transform infrared spectroscopy (FT-IR) was used to elucidate the chemical structure of the cell-bound Lp-BS by identifying type of functional groups. The spectrum indicates a glycoprotein nature for the biosurfactant.

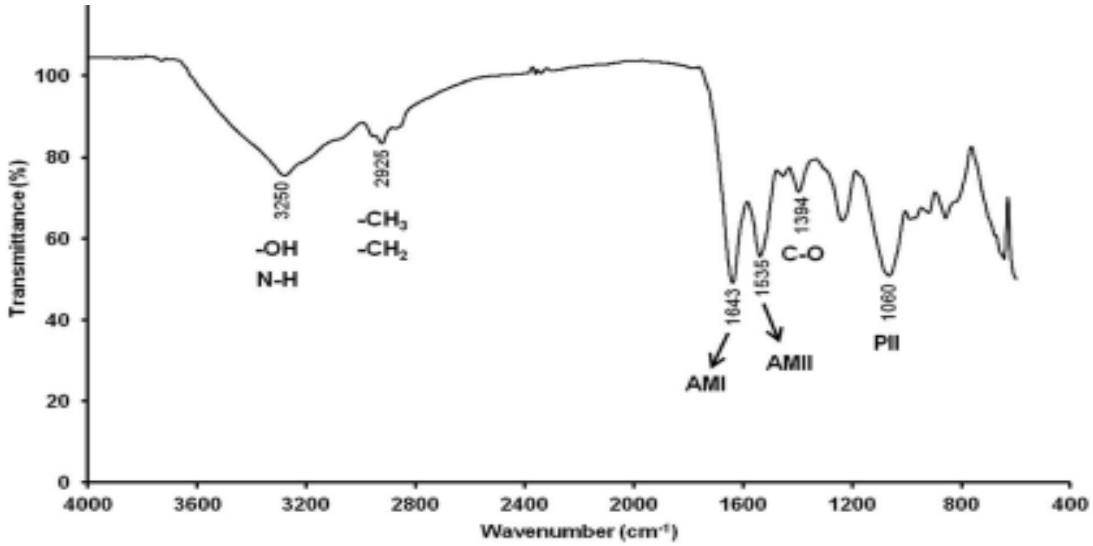


Figure 3.5 **FT-IR spectrum of cell-bound biosurfactant produced by *L. agilis* CCUG31450** published in the Royal Society of Chemistry journal (Gudiña *et al*. 2015) and supposed to be a glycoprotein. The functional group region highly matches with that of *Lp*-BS but not the fingerprint region.

3.3.3.2 Protein assay, SDS-PAGE and silver staining

3.3.3.2.1 BCA Assay

An equation was generated from a standard curve that related absorbance (y) against protein concentration (x) (Figure 3.6). The protein content of the freeze-dried PBS- and broth-extracts was calculated. The cell-bound extract sample collected at the mid-exponential phase showed the highest protein concentration of 32.6 ± 7.4 µg per 1 mg of the crude sample (Figure 3.7). The three cell-bound extract samples collected at the stationary phase time points demonstrated lower protein contents compared to the log phase sample (12.1 ± 3.7, 15.3 ± 5.1, 11.9 ± 2.4 µg/mg respectively). Protein biomasses of the broth extracts obtained at the four time points (206.16 ± 38.43, 187.24 ± 55.21, 199.5 ± 34.98, 182.3 ± 48.22 µg/mg respectively) were found to be less than the broth control (227.47 ± 44.9 µg/mg) (Figure 3.7).

After dialysis, protein content of the selected cell-bound mid-exponential time point extract (*Lp*-BS) increased to 538 ± 160 µg/mg (Table 3.2) as a result of concentrating the sample.

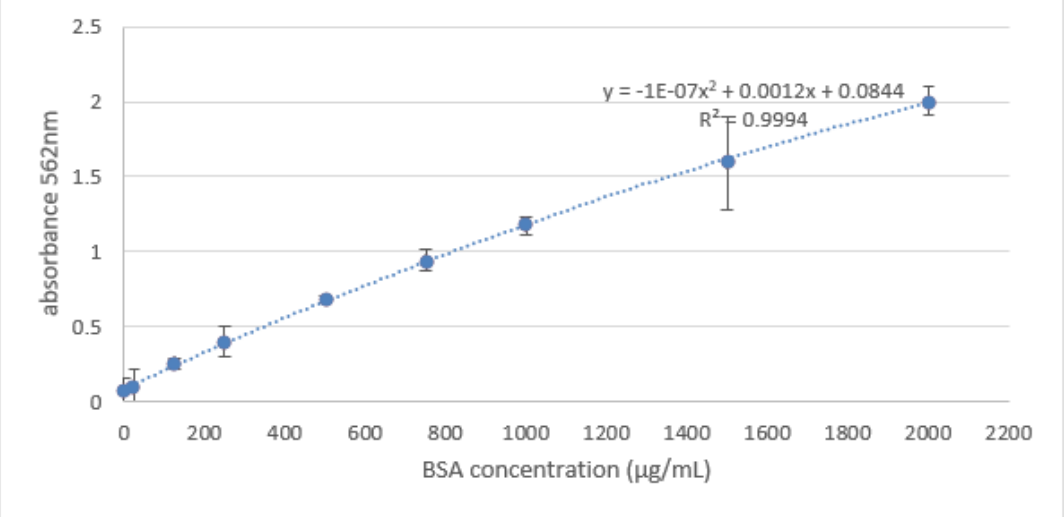


Figure 3.6 **Standard curve generated from BCA assay relating protein concentration to absorbance.** The equation of the line allowed calculation of protein concentration in the supernatants of a known absorbance.

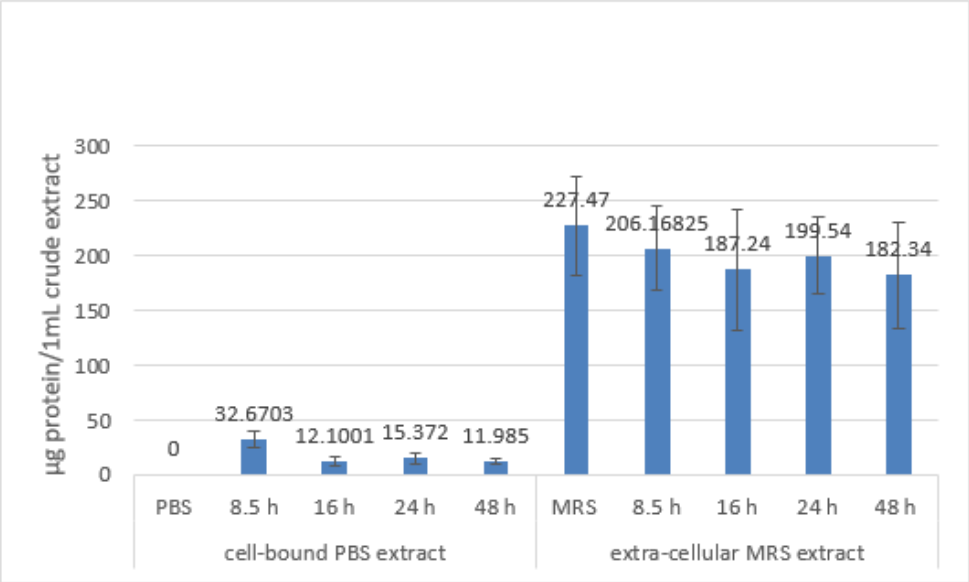


Figure 3.7 **Protein concentration of the PBS and MRS-extracts at the four time points selected for extraction.** Among the PBS-extracts, the 8.5h sample (mid-exponential one) showed approximately two-fold higher content of protein than the other three stationary phase time points. Data expressed as mean ± SD (n=3). The protein contents of the broth extracts were lower than the broth control which might be due to consumption during growth.

3.3.3.2.2 Protein Separation with Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and silver staining

The protein separation profile of the cell-bound extracts obtained at the four time points indicated the presence of a wide variety of protein components. The sample collected at the mid-exponential phase (8.5 h) showed more intense bands than samples from the other three time points (16 h, 24 h and 48 h). These results are in accordance with the BCA protein concentration result. The identified bands had a molecular weight ranging from as low as 10 KDa to high molecular weight of around 250 KDa (Figure 3.8 Left panel).

Analysing the mid-exponential cell-bound extract (*Lp*-BS) after dialysis revealed three distinct protein bands of 10, 15 and 30 kDa and a smear band at 100-250 kDa (Figure 3.8 Right panel).

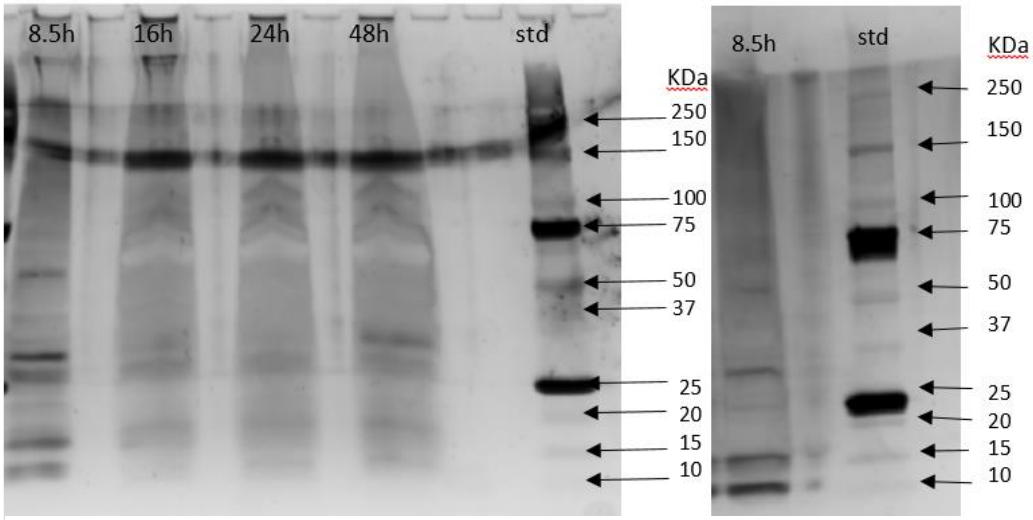


Figure 3.8 **Left: SDS-PAGE of *L. plantarum* PBS-extracts** at four time points (8.5, 16, 24 and 48h) showing that the 8.5 h sample contained more protein bands of different molecular weight compared to the samples collected at stationary phase. **Right: SDS-PAGE of the mid-exponential biosurfactant (*Lp*-BS) after dialysis**.

3.3.3.3 Thin layer chromatography (TLC)

No lipid residues were detected when the biosurfactant developed silica plate was sprayed with ammonium molybedate/serium sulphate mixture (Figure 3.9). This result indicated the absence of lipid moiety in the examined cell-bound biosurfactant (*Lp*-BS). However, rhamnolipid which was used as a positive control, gave two distinctive spots, indicative of the presence of mono and di- rhamnolipid mixture (Figure 3.9 red arrows).

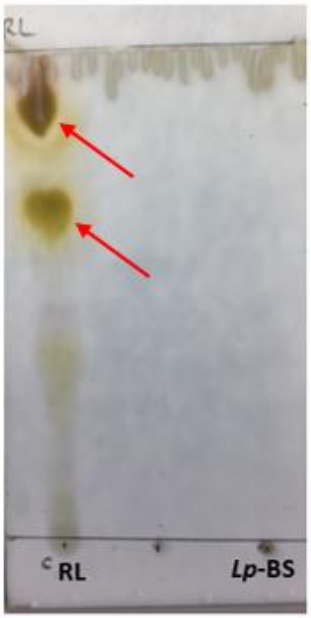


Figure 3.9 **Thin layer chromatography of *Lp*-BS**. The extracted biosurfactant *Lp*-BS demonstrated no lipid spot following staining with ammonium molybdate/cerium IV sulphate stain while the two lipid moieties of the mono- and di-rhamnolipid (RL) positive control are shown as brown spots (red arrows).

3.3.3.4 Anthrone assay

Anthrone assay for carbohydrate content of the crude biosurfactant extract (*Lp*-BS) was employed using glucose as a standard to generate the standard curve (Figure 3.10). The presence of carbohydrate by indicated with the development of a bluish-green colour observed following the incubation of the crude biosurfactant solution with anthrone reagent. The amount of the carbohydrate present was estimated using the standard graph (Figure 3.10) and a concentration of 0.186 ± 0.07 mg per 1mg of the crude sample was calculated (Table 3.2).

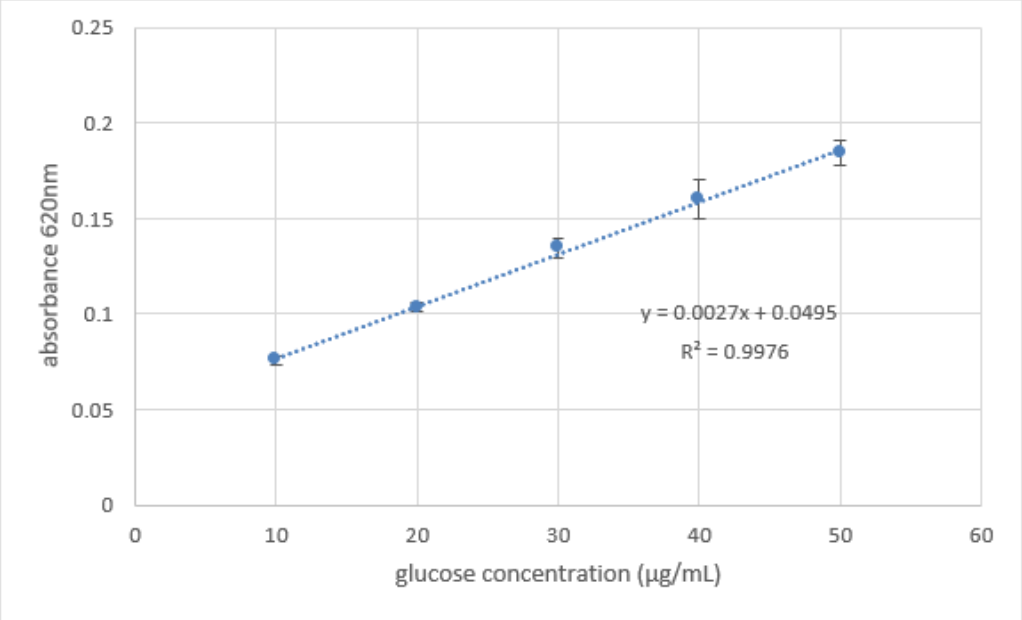


Figure 3.10 **Standard curve generated from anthrone assay** relating glucose concentration to absorbance at 620 nm. The equation from the curve y = 0.0027x + 0.0495 allowed to calculate carbohydrate concentration in the dialysed cell-bound extract.

Table 3.2 **Summary of the chemical composition of the crude biosurfactant extract (*Lp*-BS)**

Compound	mg of the compound /1 mg of dialysed cell-bound BS (*Lp*-BS)
Carbohydrate	0.186 ± 0.07
Protein	0.538 ± 0.16
Lipid	-

3.3.4 Fractionation by size exclusion and chemical analysis

The crude *Lp*-BS was fractionated by size using a fast protein liquid chromatography (FPLC). Five absorption peaks were detected (Figure 3.11). A sharp tall peak was detected for fractions A6-A9 followed by short but broad peak between A10-B14. Three small sharp peaks were observed from fractions B13-B1. Based on the chromatogram profile (Figure 3.11), fractions A6-A9, A10-B14, B13-B10, B9-B7 and B6-B1 were pooled and denoted fraction 1 (F1),

fraction 2 (F2), fraction 3 (F3), fraction 4 (F4), and fraction 5 (F5) respectively. Low yield was obtained for fractions 3-5 (1 mg/20 mg of loaded *Lp*-BS). Dialysed freeze-dried fractions were subjected to anthrone assay for the detection of carbohydrate presence. Fraction 1 (F1) and fraction 2 (F2) were positive for carbohydrate while no sugar was detected for the other three fractions (F3, F4 and F5). Analysis for protein was performed via SDS-PAGE and the gel was stained with colloidal coomassie stain (Figure 3.12). No protein bands were observed for fraction 3, 4 and 5. Conversely, many protein bands of different intensity and molecular weights were visible for fraction 1 and 2. Fraction 1 was the most abundant of protein bands of molecular weight of 10-110 KDa. Fraction 2 also revealed many protein bands of molecular weights between 10-73 KDa.

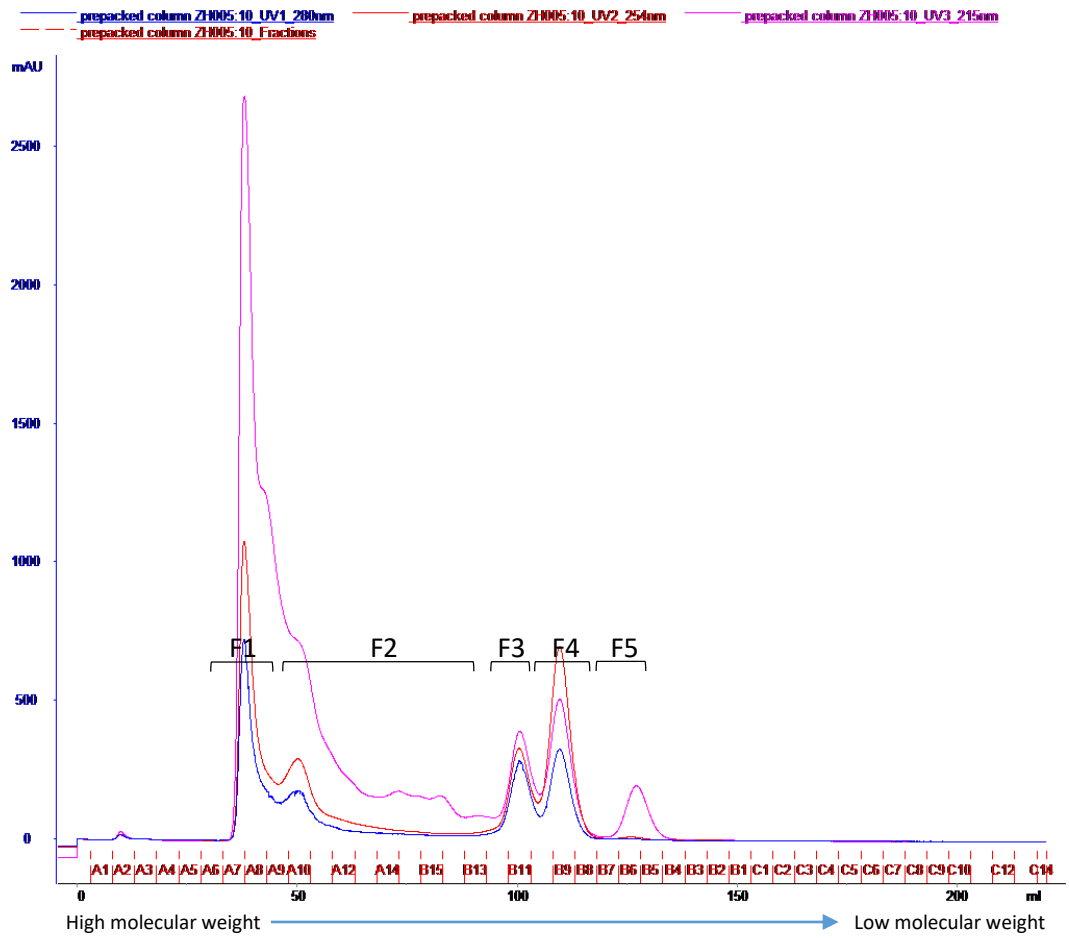


Figure 3.11 **Representative chromatogram of crude *Lp*-BS fractionation via size exclusion FPLC.** Five peaks were eluted through Sephacryl HR-100 (fractionation range 1-100 KDa). Additional FPLC runs yielded identical chromatogram profiles.

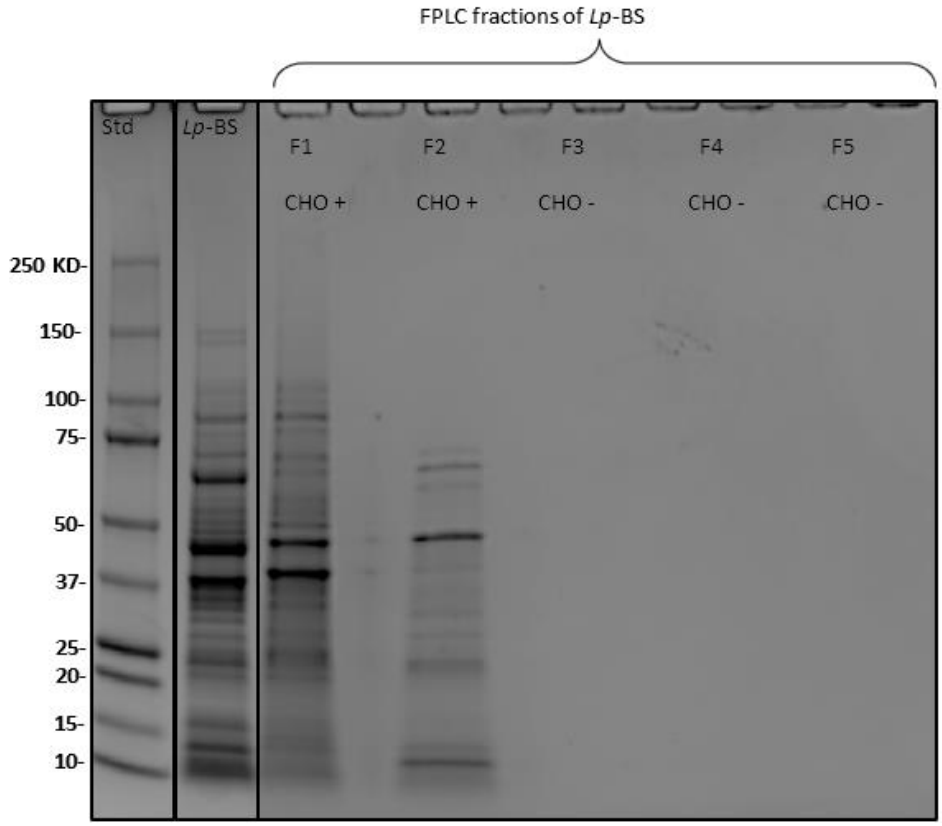


Figure 3.12 **Protein separation of the crude *Lp*-BS and its size exclusion fractions by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).** The gel was stained with colloidal coomassie stain. No bands of fractions 3, 4, and 5 (denoted F3, F4 and F5) were visible. Fractions 1 (F1) and 2 (F2) showed many bands of different molecular weights and intensity. CHO stands for carbohydrate examined by anthrone assay for the five *Lp*-BS fractions; + indicates positive for sugar, - indicates the absence of sugar.

3.3.5 Detection of biosurfactant fractions by EI24

By running an emulsification index 24h assay on the first two fractions (F1 and F2), it was shown that only fraction 1 demonstrated emulsifying activity on xylene indicating that this fraction contained the biosurfactant compound. However, fraction 2 showed similar result to the water negative control indicative of the absence of biosurfactant (Figure 3.13).

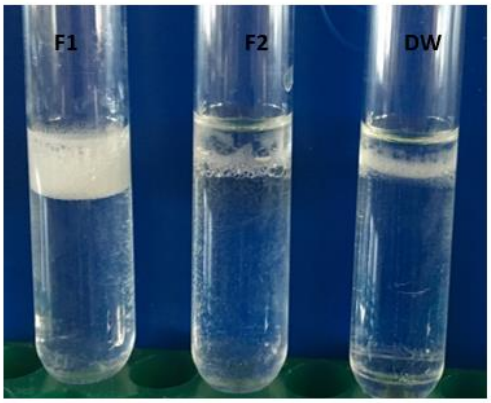


Figure 3.13 **Emulsification index 24 (EI24) of the freeze-dried FPLC fractions.** Only fraction 1 showed emulsifying activity against xylene demonstrated as a creamy white top layer. No difference between the negative water control (DW) and F2 in their emulsifying effect when mixed with xylene indicative of the absence of biosurfactant. DW, distilled water.

3.3.6 Liquid chromatography-mass spectrometry (LC-MS)

Protein separation profile of *Lp*-BS by means of SDS-PAGE and coomassie stain revealed that numerous protein bands of different intensities were detected (Figure 3.14). Their molecular weights ranged from 10-150 KDa. Ten bands (most intense ones) were cut and sent for LC-MS analysis. The reports from Bristol proteometry centre showed the results of a Sequest search against the Uniprot *L. plantarum* database. The data were filtered at 1 % FDR and also any proteins that were only matched by a single peptide were removed. The identification was performed twice and consistent results were obtained. Numerous different proteins (>100) were detected for each band (see Appendix I). A score cut-off of ≥ 100 of the identified proteins was selected to narrow down the search and the search was done using Uniprot (https://www.google.co.uk/search?q = uniprot&rlz=1C1JZAP_arGB700GB710&oq = uniprot&aqs = chrome.69i57j0l5.6499j1j4&sourceid = chrome&ie=UTF-8, 27/ Nov / 2018) and other publications of similar proteins of *Lactobacillus* spp. Bands 9 and 10 (Figure 3.14) were not included in the search because their maximum proteins scores were below the selected cut-off.

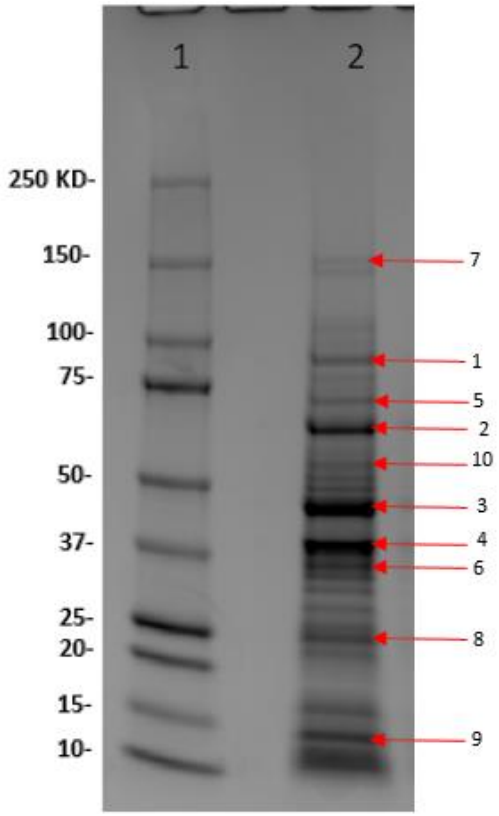


Figure 3.14 **Protein separation profile of crude *Lp*-BS in preparation for mass spectrometry**. Gel stained with colloidal coomassie stain. Ten bands (red arrows) were cut and subjected to the process of digestion in preparation of LC-MS analysis. 1, standard; 2, *Lp*-BS.

A search for *Lactobacillus* surface proteins that contribute to cell adhesion was undertaken. Only three proteins were found to be involved in *L. plantarum* adhesion (Table 3.3). Bands 2, 3, 4, and 5 (Figure 3.14) were found to contain the protein "Elongation factor Tu OS=*Lactobacillus plantarum* (strain ATCC BAA biosynthesis (Uniprot), this protein was identified as a mucin adhesion factor of *L. plantarum* CS23 and *L. plantarum* CS24·2 (Dhanani and Bagchi 2013) (Table 3.3). Likewise, Glyceraldehyde 3-phosphate dehydrogenase OS=*Lactobacillus plantarum* (strain ATCC BAA-793 / NCIMB 8826 / WCFS1) OX=220668 GN=gapB PE=3 SV=1 - [F9UM10_LACPL] (Swiss-Prot accession number F9UM10) was identified in bands 3, 4, 6, 8 (Figure 3.14) and was found to function as mucin binding protein (Kinoshita *et al.* 2008) (Table 3.3). Enolase 1 OS=*Lactobacillus plantarum* (strain ATCC BAA-793 / NCIMB 8826 / WCFS1) OX=220668 GN=eno1 PE=3 SV=1 - [ENO1_LACPL] of an accession number Q88YH3 (Swiss-Prot) was identified in band number 3 only (Figure 3.14). This protein functions as fibronectin-binding protein (Uniprot, 27/Nov/2018) and a collagen-binding protein (Castaldo *et al.* 2009; Kainulainen and Korhonen 2014; Salzillo *et al.* 2015) (Table 3.3).

Table 3.3 ***Lp*-BS mass spectrometric identification of adhesin-like proteins**

Protein	**Uniprot Accession no.**	**Band no.**	**Score** in each band respectively	**% coverage** in each band respectively	**Function***	**Reference**
Elongation factor Tu	Q88VE0	2, 3, 4, 5	104.6, 894.0, 136.5, 158.7	64.5, 74.4, 64.3, 64.5	Mucin adhesion factor	Dhanani and Bagchi 2013
Enolase 1	Q88YH3	3	252.6	69.2	Fibronectin and collagen-binding protein	Uniprot; Castaldo *et al.* 2009; Kainulainen and Korhonen 2014; Salzillo *et al.* 2015.
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	F9UM10	3, 4, 6, 8	110.8, 776.2, 311.7, 106.0	63.8, 80.2, 69.7, 63.5	Mucin-binding protein	Kinoshita *et al.* 2008

*, adhesin-like function is stated here only, other metabolic and catalytic functions are not included; Score, The sum of the ion scores of all peptides that were identified; % Coverage, The percentage of the protein sequence covered by identified peptides.

3.4 Discussion

As previously reported (Chapter 2), *L. plantarum* showed potential antimicrobial activity against SAG and *E. faecalis* growth *in-vitro* justifying further investigations. Biosurfactant production has been proposed as one of the antimicrobial mechanisms of lactic acid bacteria (Reid and Bruce 2001; Sharma *et al.* 2016). In this chapter, *L. plantarum* was assayed for biosurfactant production as a possible antimicrobial.

In this work and for the first time, a high molecular weight cell-bound biosurfactant was successfully extracted from *L. plantarum* NCIMB8826 strain at the mid-log phase by passive release into PBS. The extracted crude biosurfactant showed good surface tension and emulsifying activity comparable to that of the commercial rhamnolipid. A preliminary characterisation by FT-IR together with conventional protein, carbohydrate and lipid analysis was found to be effective in providing a rapid, but partial, chemical picture of the crude *Lp*-BS as a glycoprotein. Mass spectrometric analysis revealed a complex proteomic composition of *Lp*-BS and successfully enabled the identification of three of the adhesin-like proteins which might suggest an antiadhesive effect of this biosurfactant (Rams *et al.* 2014). Importantly, chromatographic fractionation based on size exclusion was shown to be suitable to partially purify the biosurfactant utilising its propensity to aggregate into micelle.

To account for the kinetics of *L. plantarum* biosurfactant production and its relation to bacterial growth, samples were collected at different time points of *L. plantarum* growth curve. As biosurfactant can be either cell-bound or excreted into the culture medium, *L. plantarum* production of cell-bound and excreted biosurfactants was assessed using MRS as a culture medium. No reduction in surface tension of the culture broth was observed for any of the four MRS-extracts suggesting that *L. plantarum* does not produce extra cellular biosurfactant under the conditions studied. Contrary to that, the four cell bound samples showed the ability to reduce surface tension (Table 3.1).

Biosurfactants synthesis can be either spontaneous to increase the bioavailability of nutrients for actively growing bacteria or triggered by various physical and chemical stresses as an ecological strategy to survive such as growth under condition of low concentration of nitrogen or carbon source; for example, when bacteria reach stationary phase with limitation of the nutrients and activation of quorum sensing (QS) system (Desai and Banat 1997; Bhardwaj *et*

al. 2013; Ocampo 2016; Santos *et al.* 2016). Different biosurfactants production kinetics, though not fully characterised as yet, have been reported for various bacteria (Desai and Banat 1997; Rodrigues *et al.* 2006; Gudiña *et al.* 2011). This can be classified as (a) growth-associated production; (b) production under growth-limiting conditions; (c) production by resting or immobilised cells; and (d) production with precursor supplementation (Desai and Banat 1997; Santos *et al.* 2016). The fact that *Lp*-BS was produced over the four time points of growth phases might indicate that its production followed these kinetics mentioned earlier (except d). Since *Lp*-BS was produced when *L. plantarum* grew in MRS broth in the absence of a continuous nutritional supply (such as would be provided in a bioreactor system), it might be that it was produced impulsively during the log phase and then tapered over time due to the limitation of nutrients which are essential and determinative of biosurfactant production. Cell density has been associated with biosurfactant production (Adebusoye *et al.* 2008; Ocampo 2016) and it might be that *L. plantarum* biomass reached a critical cell density which resulted in a negative feed-back reduction to biosurfactant production.

No satisfactory information is available to explain why certain bacteria produce cell-bound and others, extracellular biosurfactants. Nevertheless, it has been proposed that extracellular biosurfactants facilitate the emulsification of substrates that are insoluble in water (hydrophobic) and make them accessible for bacterial utilisation while the cell bound biosurfactants might have a role in the bacterial cell adhesion to different surfaces (Desai and Banat 1997; Tan and Kong 2000; Ocampo 2016). Accordingly, the lack of hydrocarbons in the MRS medium might explain why a cell-bound biosurfactant was produced by *L. plantarum* rather than a released extracellular one. However, given that a recent review of lactic acid bacterial species demonstrated that a majority (40 out of 46) produced cell-bound biosurfactants (Satpute *et al.* 2016) and that lactic acid bacteria (LAB) are frequently reported to play a beneficial role in preventing tissue attachment by pathogens and colonisation (Blomberg *et al.* 1993; Satpute *et al.* 2016), it seems plausible that cell-bound biosurfactant production is a species-specific phenomenon contributing to the native antiadhesive role of LAB in their natural environment. This may explain why the focus for cell associated biosurfactant usage appears to be related to anti-adhesive rather than antimicrobial effects.

Similarly, a strain of *L. plantarum* isolated from the vagina of healthy Nigerian women showed the potential to produce large amounts of cell-bound biosurfactant after 18 h incubation in

MRS medium. This strain was proposed as a good anti-infective candidate since it demonstrated inhibitory effect against urogenital pathogens (Anukam and Reid 2007). In another study (Madhu and Prapulla 2014), *L. plantarum* CFR 2194, an isolate from kanjika, a rice-based Ayurveda fermented product was tested for biosurfactant production over 24, 48 and 72 h in MRS. The three cell-bound samples (24, 48 and 72 h) demonstrated a reduction in surface tension with the maximum effect observed for the sample collected late at 72 h with significant antiadhesive activity against food-borne pathogens. Moreover, two *L. plantarum* strains (L26 and L35) isolated from Romanian traditional fermented food products grown in MRS medium were found to produce cell-bound biosurfactants during the exponential growth phase, but production continued to increase significantly in the stationary phase, and reached highest value after 48 h of incubation (Cornea *et al.* 2016).

Although *Lp*-BS was produced at a relatively low biomass of 40 mg/L of culture medium, it is in accordance with the biomass of cell-bound biosurfactants extracted from other lactic acid bacteria (20-100 mg/L) (Velraeds *et al.* 1996; Velraeds *et al.* 1996; Busscher *et al.* 1997; Rodrigues *et al.* 2006b; Rodrigues *et al.* 2006; Gudiña *et al.* 2015).

Lp-BS was shown to exert surface tension and emulsifying activity and therefore can be considered as a biosurfactant and bioemulsifier (Willumsen and Karlson 1996; Batista *et al.* 2006). A reduction of surface tension of > 8 mN/m has been considered as an indicator for biosurfactant-producing bacteria (Busscher *et al.* 1994; Velraeds *et al.* 1996). A reduction of surface tension between 12-33 mN/m by cell-bound biosurfactants extracted from different *Lactobacillus* spp (Velraeds *et al*. 1996; Meylheuc *et al*. 2006; Rodrigues, *et al.* 2006b; Gol·ek *et* al. 2009; Brzozowski *et al.* 2011; Gudiña *et al.* 2011; Madhu and Prapulla 2014) has been reported, and this is in accordance with the results obtained herein with *Lp*-BS (17.3 ± 0.5 mN/m). Comparable emulsifying activity (28 %) was reported for *Lp*-BS to that of other lactobacilli. For instance, *L. plantarum* CFR2194 was documented to produce a biosurfactant with emulsifying effect on different hydrocarbon substrates of around 13-38 % (Madhu and Prapulla 2014). Another *Lactobacillus*, *L. pentosus* CECT4023, was reported to produce biosurfactant which emulsified gasoline, kerosene and octane to a higher extent (EI24 > 40%) (Moldes *et al*. 2013). An even more powerful bioemulsifier was extracted from *L. agilis* CCUG31450 with emulsifying index of 60 % against n-hexadecane (Gudiña *et al.* 2015). In industry, particularly pharmaceutical, surfactants are extensively utilised. The use of these

synthetic products may present concerns with regard to environmental pollution and noxious hazards. Hence, biosurfactants have been studied as an alternative (Desai and Banat 1997) offering the advantages of higher environmental sustainability, better foaming properties and stability at pH, salinity and temperature extremities (Mukherjee *et al.* 2006). Since *Lp*-BS was found able to demonstrate these two surface activities, a promising application of this agent might be achieved in the pharmaceutical formulations such as the preparation and stabilisation of emulsion, the antimicrobial activity via detergent-like effect on cell membrane permeability (Tahzibi *et al.* 2004; Tabatabaee *et al.* 2005; Techaoei *et al.* 2007; Rahman and Takashi 2009) and in the cosmetic industry (Vecino *et al.* 2017).

Only limited information is available regarding the biochemical composition of the reported *Lactobacillus* biosurfactants which may be due, in part to their complex structure. Only 50 % of the publications regarding the cell-associated *Lactobacillus* biosurfactants have provided reports on their chemical composition (Satpute *et al.* 2016). Protein, carbohydrate and lipid analysis of *Lp*-BS were shown positive but not for lipid. This finding is further supported by FT-IR analysis of *Lp*-BS with functional groups which indicated the identification of a glycoprotein compound. Similarly, glycoprotein biosurfactants were identified from a number of lactobacilli; *L. plantarum* CFR2194 (Madhu and Prapulla 2014), *L. pentosus* CECT4023 (Moldes *et al.* 2013), *Lactococcus lactis* 53 (Rodrigues *et al.* 2006), *L. casei 8/4* (Gol·ek *et al.* 2009), *L. agilis* CCUG31450 (Gudiña *et al.* 2015), *L. acidophiles* (Ismaeel *et al.* 2013) and *L. acidophilus* ATCC 4356 (Shokouhfard *et al.* 2015). Of particular interest, biosurfactants isolated from *L. agilis* CCUG31450 (Gudiña *et al.* 2015) and *L. plantarum* CFR2194 (Madhu and Prapulla 2014) had FT-IR spectra of functional regions almost identical to that of *Lp*-BS, sharing the same functional groups of protein and carbohydrate. However, their fingerprint regions were not the same, indicating they are different compounds. It has been reported that the composition of protein and carbohydrate fractions of lactobacilli glycoproteins biosurfactants are affected by many factors such as the composition of the growing medium, duration, pH, temperature of incubation, inoculum volume and the growth phase of bacteria (Ch and Khanaqa 2010; Satpute *et al.* 2016). To the best of knowledge, no previous reports on biosurfactants have highlighted the value of the FT-IR fingerprint region as a differentiating tool to compare the biosurfactants of the same constituents.

The protein profile obtained via SDS-PAGE and silver staining indicated that the mid-exponential phase time point demonstrated more protein bands than in samples collected from the other time points (16, 24 and 84 h), which may be due to the physiology of the log phase when actively growing cells undergo DNA synthesis, transcription and translation to synthesise the required molecules (Faith *et al.* 2007). The abundance of protein bands reflects the heterogeneity of the proteins of the crude *Lp*-BS. Though not common, a number of studies used electrophoresis for biosurfactant identification. For instance, the Toren research group (Toren *et al.* 2001) successfully purified three Alasan bioemulsifier proteins and identified them by means of SDS-PAGE and coomassie stain. Their molecular weights were found to be 16, 31, and 45 kDa; the 45 KDa protein was the most surface active one. Moreover, SDS-PAGE analysis of a semi-purified biosurfactant extracted from the marine Vibrio spp. designated strain 3B-2 revealed the presence of miscellaneous proteins bands. Following its purification by chromatography, the electrophoresis revealed a pure product with single protein band gel (Hu *et al.* 2015). These findings suggest the usefulness of protein electrophoresis as an indication of purity and content of the investigated biosurfactants. In a study conducted by Shokouhfard and co-workers (Shokouhfard *et al.* 2015), a crude cell-bound biosurfactant was extracted from *L. acidophilus* ATCC 4356. Its FT-IR analysis appeared to be mostly protein. Interestingly, its protein profile obtained from SDS-PAGE and staining with coomassie blue G250 revealed the presence of a single band with approximate size of 10 kDa. Although the biosurfactant was extracted in the same way used in our study with *Lp*-BS (i.e. by suspending the cells in buffer and allow the biosurfactant to be released by gentle stirring), there was no details on the electrophoresis and staining procedures. The use of different procedures might impact on the separation of biosurfactant proteins.

Further analysis of the crude *Lp*-BS by in-gel mass spectrometry identified a large number of proteins, of which, three (enolase, glyceraldehyde-3-phosphate dehydrogenase and elongation factor) have been identified as adhesin-like proteins in addition to having other metabolic or catalytic activities, thus can be termed as “moonlighting” proteins (Kainulainen and Korhonen 2014). Host tissue extracellular matrix proteins can act as a target for pathogen interaction and subsequent tissue colonisation via specific bacterial surface molecules (Chagnot *et al.* 2012; Singh *et al.* 2012). Likewise, probiotics can display cell surface molecules that specifically interact with the host tissue receptors and competitively exclude the

pathogens from binding to the same receptor sties thereby exerting their beneficial effects (Remus *et al.* 2013; Salzillo *et al.* 2015). Such host tissue surface molecules include collagen, laminin, fibronectin, mucin and fibrinogen (Castaldo *et al.* 2009; Sengupta *et al.* 2013; Yadav *et al.* 2013; Vastano *et al.* 2014). A number of *Lactobacillus* adhesion proteins have been identified and considered as a critical element contributing to the beneficial effects exerted by the bacteria (Nishiyama *et al.* 2016).

A surface displaced alfa-enolase of *L. plantarum* LM3 has been characterised as a fibronectin binding protein (Castaldo *et al.* 2009). Another study revealed the involvement of EnoA1 alfa-enolase of *L. plantarum* LM3 in type I collagen (CnI) binding (Salzillo *et al.* 2015). Similarly, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been found to be involved in the probiotic *L. plantarum* LA 318 adhesion to human colonic mucin. Possessing GAPDH adhesin similar to that of many pathogens makes it possible for the *Lactobacillus* to compete specifically with pathogenic bacteria for adhesion sites in the mouth or intestine host tissue. The author suggested the usefulness of *L. plantarum* LA 318 for the development of probiotic anti-infective therapy (Kinoshita *et al.* 2008). In a study that investigated the role of elongation factor-Tu in *Lactobacillus* adhesion, *L. plantarum* CS23 and *L. plantarum* CS24.2 have shown the potential to adhere to mucin *in-vitro* and competitively block the adhesion of *E. coli and Salmonella enterica serovar Typhi* to mucin (Dhanani and Bagchi 2013). The authors also demonstrated an up-regulation of *Lactobacillus* EF-Tu gene upon exposure to mucin. This adhesin EF-Tu was found to significantly hinder the adhesion of enteropathogens to mucin (Dhanani and Bagchi 2013). Accordingly, the identification of these three surface adhesins in *Lp*-BS mixture might suggest a potential role of *Lp*-BS as antiadhesive biomolecules of therapeutic significance.

Due to the complex, heterogeneous protein rich nature of *Lp*-BS, an attempt to purify it was performed via FPLC. Five elution fractions were obtained (Figure 3.11). Fraction 1 and 2 were assessed for emulsifying activity and only fraction 1 was found to have emulsifying potential against xylene indicating that the crude *Lp*-BS was successfully partially purified with the aid of size exclusion chromatography. It is known for biosurfactants to form molecular aggregates termed as “micelles” above a certain concentration known as the critical micelle concentration (CMC) (Liu *et al.* 2013). This phenomenon has been utilised for biosurfactant purification based on size exclusion. Although the CMC of *Lp*-BS has not been recorded in the

current study, it is possible that *Lp*-BS formed micelles and that, due to their bulky structure, they were eluted in the first column elution fraction (F1). While other small molecular contaminants were obtained in later column fractions due to their smaller size and failure to form aggregated macromolecules. Similarly, a lipopeptide biosurfactant, extracted from the cell-free broth of a marine *Bacillus circulans* culture, was successfully purified from other contaminants via size exclusion chromatography utilising the micelles-forming nature by loading the lipopeptide biosurfactant at a concentration higher than its CMC (Mukherjee *et al.* 2009). A study conducted by Sen and Swaminathan, 2005 (Sen and Swaminathan 2005) also exploited the micelle-forming behaviour of biosurfactants to purify surfactin. However, instead of using size exclusion chromatography, they performed trans-membrane separation. Basically, under nitrogen pressure, the surfactin-containing solution was poured into a cell and a magnetic bar was continuously stirred driving the solvent and other contaminants out through the cell membrane leaving behind the supramolecular surfactin micelle.

The coomassie stained gel of the obtained *Lp*-BS FPLC fractions demonstrated the presence of considerable number of proteins for F1 indicating that the collected biosurfactant-containing fraction was only partially purified and further purification by other techniques may be required. It might be worth mentioning here that colloidal coomassie stain of the SDS-PAGE gel resulted in better resolution and highlighting of bands of the separated crude *Lp*-BS proteins than silver staining. This might be due to the glycoprotein nature of *Lp*-BS. Although silver stain can be used for protein with limited degree of glycosylation especially when extra sensitivity is required, however this stain might lose its sensitivity when applied for the detection of highly glycosylated proteins (protein-glycosaminoglycans or glycoproteins). This is presumably due the steric intervention with silver ions binding caused by the carbohydrates resulting in weak or even no detection giving a smear rather than a definite band (Møller and Poulsen 2009).

3.5 Conclusion

In summary, the work reported in this chapter was successful in the characterisation of a cell-bound glycoprotein biosurfactant extracted from the potential probiotic *L. plantarum* during its exponential growth. This biosurfactant, besides reducing surface tension of PBS, demonstrated emulsifying activity against a number of hydrocarbons making it suitable for future pharmaceutical applications. Partial purification has been achieved by performing size-

exclusion chromatography with the biosurfactant eluted in the early column fraction indicating the formation of bulky biosurfactants molecules. The heterogeneity of proteins obtained in the biosurfactant fraction signifies the need for further purification. Purification of the crude sample should be carefully analysed as each purification step will increase the costs and decreases the amounts of biosurfactants recovered. Proteins identification of *Lp*-BS by means of mass spectrometry revealed the presence of three potential moonlighting adhesins which might be involved in the biosurfactant antiadhesive activity. However, further investigations are needed to assess their role in adhesion and subsequent biotherapeutic application.

4 Chapter 4 Antimicrobial and antiadhesive activity of *Lp*-BS and rhamnolipid

4.1 Introduction

Initial screening of five probiotic stains for their antimicrobial effects against SAG and *E. faecalis* (Chapter 2) demonstrated *L. plantarum* as the potential one showing significant growth inhibitory effect against the four endodontic pathogens. *L. plantarum* was further investigated for biosurfactant production (Chapter 3) and was shown able to produce a cell-bound biosurfactant (*Lp*-BS), which was partially purified and characterised as a mixture of glycoproteins. Three proteins of adhesin-like effect were also identified in the biosurfactant mixture proposing for possible antiadhesive potential. Before translation into clinical use becomes possible, this biosurfactant compound needs to be studied further for its *in-vitro* antimicrobial and antiadhesive effect against the endodontic pathogens.

Microbial decontamination is a crucial key element for the success of vital pulp therapy and for the preparation of root canals prior to filling (Ghoddusi *et al.* 2014). This can be achieved not only by providing direct antimicrobial effect but also via prophylactic protection against microbial pulp and root canal colonisation. Such properties may be an inherent property of biosurfactants. The expanding problem of antibiotic resistance necessitates the investigation of alternative approaches to fight pathogenic infections. Generally, microbial biosurfactants have been studied for their medical and industrial applications (Singh and Cameotra 2004; Rodrigues *et al.* 2006; Oelschlaeger 2010) and their structure-related characteristics have been a field of increasing interest in exploring their biomedical applications such as antiviral, antimicrobial, antifungal and anticancer properties (Fiechter 1992; Singh and Cameotra 2004; Kosaric and Sukan 2014; Fracchia *et al.* 2015; Callaghan *et al.* 2016). A number of biosurfactants have been documented with known antimicrobial activity including surfactin and iturin produced by *B. subtilis* strains (Ahimou *et al.* 2000), mannosylerythritol lipids from *Candida antarctica* (Rufino *et al.* 2011), rhamnolipids from *P. aeruginosa* (Maier and Soberon-Chavez 2000), and biosurfactants isolated from *S. thermophilus* A and *Lactococcus lactis* 53 (Rodrigues *et al.* 2006). The specific mechanisms of biosurfactants antimicrobial activity have not been fully elucidated yet. Studies have suggested that biosurfactants have comparable mechanisms to the conventional antibiotics (Banat *et al.* 2010), for instance, they

can directly integrate into and disrupt the integrity of the pathogens cell membrane or by interfering with protein synthesis resulting in cell death (Abalos *et al.* 2001; Das *et al.* 2008; Sang and Blecha 2008; Yount and Yeaman 2013).

The ability of microorganisms to adhere to distinct host tissue surface components via binding-proteins has been considered an important virulence factor that participates in initiating infections (Flock 1999) and biofilm formation (Huang *et al.* 2011). Biosurfactants have been shown to provide another valuable application as antiadhesive agents. These molecules can adsorb to the substratum surfaces or infection sites and the process appears to result in change in hydrophobicity and/or to interfere with the microbial adhesion process (Rodrigues *et al.* 2006).

Often present as part of the normal human microbiota , probiotic bacterial species and their biosurfactants might offer the advantage of being innocuous to humans and can be effectively medically applied (Sharma *et al.* 2016). Moreover, because of their proteinaceous nature, most biosurfactants produced by strains of *Lactobacillu*s are commonly supposed to be surlactin type with high potential toward hampering pathogen adherence. Researchers have recently focused on the antiadhesive and antibiofilm properties of lactobacilli-derived biosurfactants which have provided promising results (Gudiña *et al.* 2010; Satpute *et al.* 2016).

Several well-known and widely used antimicrobial activity bioassays can be employed such as disk-diffusion, well diffusion and broth or agar dilution. More sophisticated techniques that provide rapid results such as flow cytofluorometric and bioluminescent methods are also applicable but not commonly used due to the need of specific equipment and protocol optimisation (Balouiri *et al.* 2016). Regarding antiadhesive assay protocols, pre-adhesion treatment of surfaces to be studied with the biosurfactant solution and crystal violet staining is the most commonly used method (Ahimou *et al.* 2000; Gudiña *et al.* 2010; Rufino *et al.* 2011; Janek *et al.* 2012; Gudiña *et al.* 2015). Confocal laser scanning microscopy and SEM have been also used for visualising the attached bacterial layer in the absence or presence of the biosurfactant preconditioning (Janek *et al.* 2012; do Valle Gomes and Nitschke 2012; Sambanthamoorthy *et al.* 2014; Silva *et al.* 2017; Ceresa *et al.* 2018).

In light of these and other observations and considering there are no previous studies on the effectiveness of *L. plantarum* NCIMB 8826 biosurfactant and rhamnolipid on SAG and *E. faecalis* growth or adhesion, the aim of this work presented in this chapter was to assess the *in-vitro* antimicrobial and antiadhesive efficacy of the two biosurfactants. In doing so, their use as a model antimicrobial for future application as pulp and/or root canal therapy could be considered.

4.2 Material and methods

4.2.1 Antimicrobial activity determination

4.2.1.1 Growth inhibition on agar plates by well diffusion assay

Overnight broth cultures of *S. anginosus*, *S. constellatus*, *S. intermedius* and *E. faecalis isolates* in BHI were prepared and adjusted to OD 600 of 0.08-0.1 (1.5 x 10^8 CFU/mL). Bacterial lawn plates of each studied strain were prepared by evenly swabbing the adjusted bacterial culture over the surface of an FAA plate using a sterile cotton swab. Wells were then punched into the agar using a sterile 6 mm biopsy punch. Two hundred microliter of pH 7.0 *Lp*-BS solution in PBS (50 mg/mL) or rhamnolipid (Sigma 90% purity) (50, 25 and 12.5 mg/mL in PBS) were then pipetted into the wells and the plates were incubated at 37 °C in 5 % CO_2 for 24 h. Growth of bacteria was considered to be inhibited where a clear area of agar was observed around the inoculation site, in contrast to the bacterial growth covering the rest of the plate. For the purpose of comparison with the literatures regarding the activity of the studied biosurfactants, *E. coli* NCTC 10418 and *S. aureus* NCTC 8325 were also included herein as reference strains for the Gram negative and Gram positive bacteria respectively. Their lawn plates were prepared in the same way as with the test strains. Vancomycin (1 mg/mL) was used as a positive control against the Gram positive stains and gentamicin (1 mg/mL) against the Gram negative stain, while PBS was inoculated as a vehicle control. Three plates were inoculated for each strain a time and the whole experiment was repeated on three separate occasion.

4.2.1.2 Determination of the minimum inhibitory concentration (MIC) of *Lp*-BS and rhamnolipid

The minimum inhibitory concentration of *Lp*-BS and rhamnolipid against the endodontic pathogens (SAG and *E. faecalis* RB17) and against the reference strains (*S. aureus* NCTC 8325 and *E. coli* NCTC 10418*)* was determined by broth microdilution susceptibility assay (Magalhães and Nitschke 2013) in 96-well plastic tissue culture plates (SARSTEDT, Germany). The biosurfactant was dissolved in BHI medium at a starting concentration of 50 mg/mL (pH 7.0) and sterilised by filtration through a 0.22 µm pore-size filter (Whatman, GE Healthcare, UK). Subsequently, 200 µL of BHI medium containing the biosurfactant were placed into the first column of the 96-well microplate, and 100 µL of un-inoculated BHI medium in the remaining wells. After that, 100 µL from the first column were transferred to the second

column and mixed. Serially, 100 µL was transferred to the subsequent wells, discarding 100 µL of the mixture in the final column. This process resulted in two-fold serial dilutions of the biosurfactant, with the biosurfactant concentration ranging from 50-0.01 mg/mL. The last two columns did not contain biosurfactant and served as growth and negative controls. All the wells (except for the broth negative control column) were inoculated with 10 µL of a pre-culture of the corresponding microorganism grown overnight in BHI medium at 37 °C and 5 % CO_2 and diluted to an optical density (600 nm) of 0.08-0.1 (corresponding to $1x10^8$ CFU/mL) and further diluted 1:10 in BHI (ESCMID) 2003). The absorbance (at 600 nm) of the wells was measured at time zero (T0) using a microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). The microplate was then immediately covered and incubated for 24 h at 37 °C and 5 % CO_2. After 24 h of incubation, the absorbance of each well plate (at 600 nm) was measured (T24). The MIC was determined at the concentration where the percentage of inhibition was > 90 % (Kolarević *et al.* 2016) according to the following formula:

$$\% \text{ Growth Inhibition } _c = [1 - (Ac / A_O)] \times 100$$

, where Ac represents the absorbance of the well with a biosurfactant concentration c, and A_O the absorbance of the growth control well (without biosurfactant).

Triplicate assays were performed for each microorganism and biosurfactant concentration.

4.2.2 Pre-coating treatment characterisation

To investigate for biosurfactant antiadhesive activity, three different surfaces were first examined to select for the one which could be successfully coated. These surfaces included glass slide, acrylic discs and dentine discs.

4.2.2.1 Glass surface

4.2.2.1.1 Coating glass slide with *Lp*-BS solution

Briefly, 200 µL of *Lp*-BS at a concentration of 50 mg/mL and 20 mg/mL in PBS was added to the corresponding well of an 8-well chamber soda-lime glass slide (8 Well Falcon Culture Slide, Corning Incorporated Life Sciences, USA) and the same volume of PBS buffer were added to the control wells in triplicate. The slide was then wrapped in parafin foil and incubated at 37 °C for 24 h and subsequently washed twice with PBS buffer for subsequent characterisation. The experiment was repeated three times.

4.2.2.1.2 Fluorescent characterisation of *Lp*-BS coating

After coating the glass slide with the biosurfactant solution and washing twice with PBS (as described in Section 4.2.2.1.1), its plastic chamber was removed according to the manufacturer's instructions (Falcon 8 Well Culture Slide, Corning Incorporated Life Sciences, USA) by means of the provided Chamber Removal Key and Safety Removal Fixture (Figure 4.1). The coated and the uncoated control wells were then prepared for microscopy analysis by staining with 20 µL/well of the ready to use fluorescent SYPRO Ruby Protein Gel Stain (Thermo Fisher Scientific, UK) for 1 min. The stained slide was then overlaid with a glass cover slip and visualised using Olympus Provis AX 70 fluorescence microscope. The Live/Dead filter cube was used with the fluorescent microscope because it has emission spectrum similar to that of SYPRO Ruby Protein Gel Stain so it can be used to visualise the stain and the bacteria during subsequent antiadhesive assay. A low magnification of 20x was used to get a complete view over the entire well surface area. The slides were protected from exposure to light between imaging by foiling with aluminium foil.

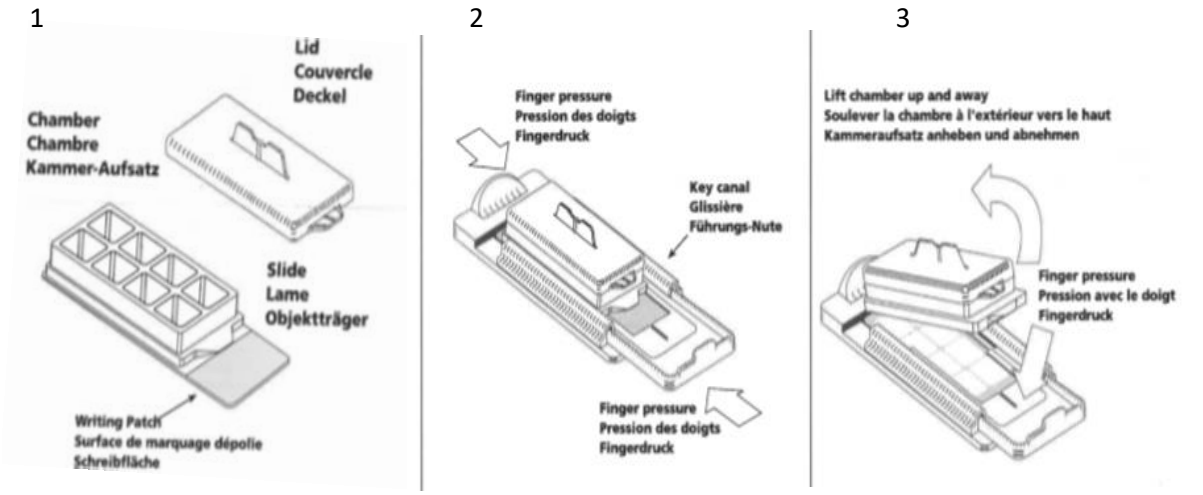


Figure 4.1 **Representative diagram of 8-well glass slide chamber removal**. Adopted from the manufacturer's instruction leaflet (Falcon 8 Well Culture Slide, Corning Incorporated Life Sciences, USA).

4.2.2.2 Acrylic (poly (methyl-methacrylate), PMMA) coupon

4.2.2.2.1 Acrylic (poly (methyl-methacrylate), PMMA) coupon preparation

To provide cheap and easy to prepare surface for coating characterisation, disc shaped poly (methyl methacrylate) (PMMA) coupons were prepared according to the manufacturer's instructions. Briefly, self-cure acrylic polymer (Bracon Ltd., Etchingham, UK) was added to self-cure acrylic monomer (Bracon Ltd.) at a 2:1 ratio and mixed by hand until homogenous. The mixture was then carefully poured into a plastic mould (with pre-cut circular sections of approximately 10 mm diameter and 2 mm thickness) and compressed between two thick and smooth glass plates. The mixture was allowed to cure under ambient conditions overnight. The coupons were sanded to remove rough edges, if present. The coupons were allowed to soak in filtered sterilised water for a minimum of 7 d to allow excess monomer to leech out, before being sterilised by autoclaving at 121 °C for 15 min. Coupons were stored in sterile distilled water prior to use, and as they were intended for single use only, used coupons were discarded after experimentation.

4.2.2.2.2 Coating acrylic coupons with rhamnolipid and *Lp*-BS solutions

Solutions of rhamnolipid (0.048, 0.097 and 50mg/mL) and *Lp*-BS (50 mg/mL) in PBS were prepared. An appropriate number of sterile acrylic coupons prepared (as described above) were placed in each bijoux bottle containing the rhamnolipid, *Lp*-BS solution or PBS buffer as a negative control and incubated at 37 °C with continuous agitation for 24 h. Subsequently the coupons were removed from the biosurfactant solution and PBS and washed twice with PBS for subsequent characterisation.

4.2.2.2.3 Characterisation of rhamnolipid and *Lp*-BS-acrylic coupon coating with scanning electron microscopy (SEM)

Scanning electron microscopy was used to further characterise the biosurfactant-coating of acrylic coupons. The pre-coated coupons were coated (as described above) and allowed to air-dry for 15 min. The coupons were sputter coated with gold (2-20 nm thick) using sputter coaters with rotary pump and gold-palladium target (DSR1 desk sputter coater; VacTechniche- East Sussex, UK), and imaged on a Tescan VAGA SEM system, at 5-10 kV.

4.2.2.2.4 Characterisation of rhamnolipid-acrylic coupons coating with crystal violet staining

Crystal violet stain binds to the carbohydrates and peptidoglycans in bacterial cell walls (Beveridge 2001). This property was utilised here for the characterisation of rhamnolipid coating where it can bind the rhamnose moiety. Briefly acrylic coupons were coated with 50 mg/mL rhamnolipid as previously outlined in section 4.2.2.2.2. The discs were then allowed to air-dry and then stained with Gram's stain crystal violet (Thermo Scientific™ Remel™, Gram Stain Kit) for 5 min. The coupons were then removed from the stain and washed once with running tap water and examined by eye in comparison to the uncoated discs.

4.2.2.3 Dentine discs

4.2.2.3.1 Dentine discs coating with rhamnolipid and *Lp*-BS solutions

Solution of rhamnolipid and *Lp*-BS in PBS were prepared at concentrations of 50 mg/mL and 20 mg/mL respectively. Three dentine discs (5mm diameter wafers of devitalised dentine, IDS, immunodiagnostic systems) were placed in each bijoux bottles containing the rhamnolipid solution, *Lp*-BS solution or PBS buffer as a negative control and incubated at 37 °C with continuous agitation for 24 h. Subsequently the discs were removed from the biosurfactants solutions or PBS and washed twice with PBS for subsequent characterisation.

4.2.2.3.2 Characterisation of rhamnolipid and *Lp*-BS coating with scanning electron microscopy (SEM)

To characterise the biosurfactant coating, the coated dentine discs (described above) were allowed to air-dry for 15 min. The discs were then sputter coated with gold, and imaged on a Tescan VAGA SEM system, at 5-10 kV as previously described (Section 4.2.2.2.3).

4.2.3 Antiadhesive activity

4.2.3.1 *Lp*-BS-coated glass slides

4.2.3.1.1 Evaluation of *Lp*-BS antiadhesive effect via microscopic imaging

To investigate for the potential of the biosurfactant coating to interfere with the attachment of endodontic pathogens SAG and *E. faecalis* to glass surface, an antiadhesive assay was performed. An overnight bacterial culture was prepared in BHI, pelleted and adjusted to an optical density (600 nm) of 0.6. An aliquot of 200 µL of the adjusted bacterial suspension was added to each well precoated with 20 mg/mL *Lp*-BS (as previously outlined in Section

4.2.2.1.1) and the same volume of PBS was added to the control wells. The slide was then incubated for 2 h at 4 °C. At the end of the incubation period, each well content was discarded and unattached microorganisms were removed by washing the wells once with 200 µL PBS. The adherent microorganisms were then fixed with 200 µL 4 % (v/v) formalin (Fisher, UK) for 24 h for subsequent imaging. Propidium iodide stain (*Bac*Light™ Bacterial Viability Kit, Life Technologies) was prepared according to the supplier's instructions at a ratio of 1 µL in 1 mL PBS and protected from light. The slide chamber was removed (as discussed in Section 4.2.2.1.2, Figure 4.1) and the fixed cells were then stained with 10 µL of propidium iodide per well and overlaid into a glass cover slip. The slide was then examined by CLSM (Leica SP5, AOBS spectral confocal microscope, Leica, Heidelberg, Germany) using the appropriate excitation and emission spectra of the Live/Dead stain (excitation 470 nm, emission 490–700 nm) (*Bac*Light™ Bacterial Viability Kit, Life Technologies) at 40x objective lens. Images of 5 areas on each well were obtained.

4.2.3.1.2 Semi-quantification of CLSM microscopic images using COMSTAT

The surface area covered by the fluorescent bacteria was determined using the image analysis program comstat2. Leica image files (.LIF) were converted to OME-TIFF files for analysis by COMSTAT using the BIOSTAT macro provided with the software, in ImageJ. The software splits files by colour, generating an image for the red (dead) channel. These images are converted to greyscale, and any pixel above the threshold value was included for analysis. The area covered by the fluorescent bacteria was then measured and calculated.

4.2.3.1.3 Evaluation of *Lp*-BS antiadhesive effect via viable cell count

In addition to microscopical analysis, a viable cell count of the recovered cells from coated wells was also performed. Ten and 20 mg/mL concentration of *Lp*-BS was used for glass surface coating as described earlier (see Section 4.2.2.1.1). An aliquot of 200 µL bacterial suspension in PBS adjusted to an optical density (600 nm) of 0.6 was added to each well precoated with either 10 or 20 mg/mL *Lp*-BS and the same volume of PBS was added to the control wells. The slide was then incubated for 2 h at 4 °C. At the end of the incubation period, each well content was discarded and the unattached microorganisms were removed by washing the wells once with 200 µL PBS. The slide chamber was removed carefully according to the manufacturer's instructions (as explained in Section 4.2.2.1.2, Figure 4.1) and a 25 cm cell scraper (Sarstedt, USA) was used to harvest attached bacterial cells into centrifuge tubes

containing 1 mL sterile PBS. The bacterial suspension was then vortex mixed and 10-fold serially diluted to 10^{-4} in sterile PBS. Fifty µL of each bacterial dilution were spirally plated onto FAA plates using a spiral plater (Don Whitley Scientific Limited, Shipley, UK). The inoculated agar plates were incubated at 5 % CO_2 and 37 °C for 24 h until colonies had grown sufficiently to allow visual counting of individual colonies.

4.2.3.2 Rhamnolipid-coated acrylic coupons

4.2.3.2.1 Evaluation of rhamnolipid antiadhesive effect via microscopic imaging

The antiadhesive propensity of rhamnolipid-coating of acrylic coupons was assayed. Briefly, coupons were coated with different concentrations (50, 0.097 and 0.048 mg/mL) of rhamnolipid solution and uncoated coupons were served as negative controls (as described in Section 4.2.2.2.2). Each of the coated or uncoated coupon was gently transferred into individual wells of 24-well plate (Sarstedt, USA) and 1 mL of bacterial suspension in PBS (adjusted to OD_{600}=0.6) was added to each well. The plate was then incubated for 2 h at 4 °C. At the end of the incubation period, the coupons were then transferred into individual wells of 24-well plate containing 1 mL PBS for washing and then into wells containing 1 mL of 4 % formalin (Fisher) for fixation for 24 h. Each of the fixed coupon was then inverted into a glass cover slip containing 10 µL propidium iodide (1 µL propidium iodide in 1 mL PBS). The coupon surface was examined by CLSM (Leica SP5, AOBS spectral confocal microscope, Leica, Heidelberg, Germany) using the excitation and emission spectra of the Live/Dead stain (excitation 470 nm, emission 490-700 nm) (*Bac*Light™ Bacterial Viability Kit, Life Technologies) at 40x objective lens. Images of 5 areas on each coupon were obtained and analysed by Comstat software as outlined in section 4.2.3.1.2

4.2.3.2.2 Evaluation of rhamnolipid antiadhesive effect via viable cell count

Viable cell counts of the recovered bacterial cells was performed alongside microscopic analysis. The effect of 50, 0.097 and 0.048 mg/mL rhamnolipid-acrylic coupons coating on SAG and *E. faecalis* adhesion on acrylic discs was assessed by determining the viable bacterial cell counts. The same procedure as explained earlier in section 4.2.3.2.1 was followed, but instead of fixation in formalin, the coupons were transferred into sterile plastic bijoux bottles containing 5 mL PBS and subjected to vortex at 2500 rev/min for 30 sec to detach bacteria. The resulting bacterial suspension was then 10-fold serially diluted in PBS to 10^{-4}. For each dilution, 50 µL were spirally plated (Don Whitley Scientific Limited, Shipley, UK) on FAA plates

and incubated for 24-48 h at 5 % CO_2 and 37 °C and colonies counted. Results were expressed as $\log_{10}$ CFU per mL for three independent experiments.

4.2.4 Statistical analysis

All experiments were subjected to three repeats on separate occasions. Un-paired t-test (GraphPad InStat 3 (v3.06)) was used to evaluate statistical difference between tests' means to the untreated control of COMSTAT images of *Lp*-BS antiadhesive assay. Dunnett's one way ANOVA (GraphPad InStat 3 (v3.06)) was used to assess statistical difference between the tests' means to the untreated control of viable count antiadhesive assay of *Lp*-BS and rhamnolipid and for COMSTAT images of rhamnolipid antiadhesive assay. A p value of ≤ 0.05 was considered significant (*, $p \leq 0.05$), very significant (**, $p \leq 0.01$) or highly significant (***, $p \leq 0.001$).

4.3 Results

4.3.1 Antimicrobial activity of *Lp*-BS and rhamnolipid

4.3.1.1 Zones of inhibition on agar well diffusion assay

Figure 4.2 shows representative images of the results obtained, which were comparable for all 3 independent experiments. Wells that had been inoculated with the three rhamnolipid concentrations (50, 25 and 12.5 mg/mL) showed a zone of inhibition against the three SAG members and *S. aureus* growth. *E. faecalis* and *E. coli* growth was not affected by the rhamnolipid demonstrated by no zone of inhibition after 24 h incubation (Figure 4.2). Yellowish zones indicated the haemolysis of red blood cells of the FAA agar medium caused by rhamnolipid. *Lp*-BS on the other hand, did not show any antimicrobial activity against any of the tested pathogens indicated by no zone of inhibition at the high concentration of 50 mg/mL (Figure 4.2).

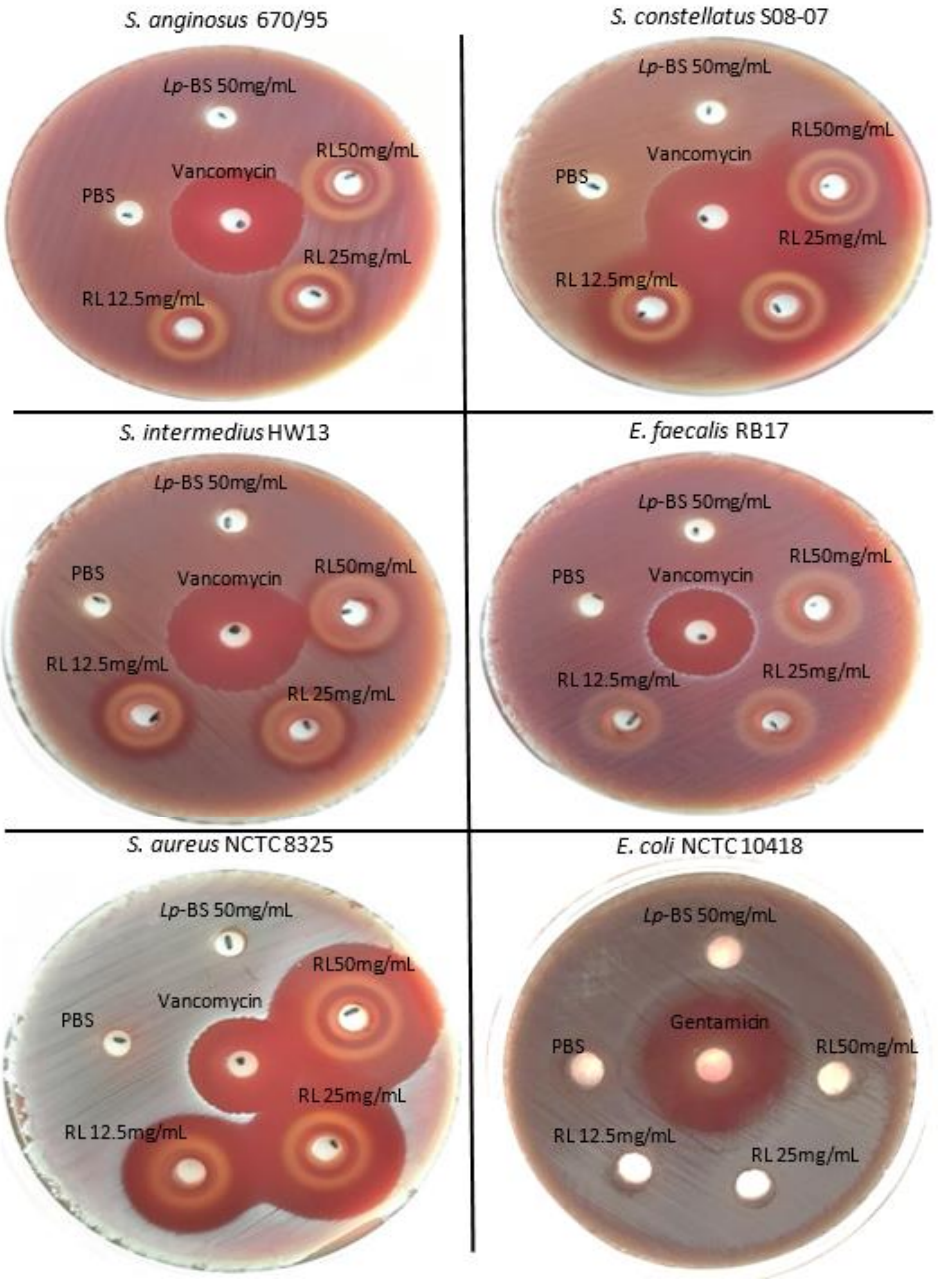


Figure 4.2 **Agar well diffusion assay with rhamnolipid and *Lp*-BS.** FAA plates were swabbed with culture of SAG members, *E. faecalis*, *S. aureus* or *E. coli*. Wells were inoculated with the corresponding material and cultured for 24 h. No antimicrobial activity was detected for *Lp*-BS (50 mg/mL) against any of the tested pathogens. Rhamnolipid (RL) demonstrated zone of inhibition against SAG members, *S. aureus* but not *E. faecalis* and *E. coli.* PBS, phosphate buffer, RL; rhamnolipid. Images are representative of three independent repeats.

4.3.1.2 Minimum inhibitory concentration determination

4.3.1.2.1 Rhamnolipid minimum inhibitory concentration (MIC)

Bacteria were incubated for 24 h with rhamnolipid solutions in order to assess the antimicrobial efficacy of rhamnolipid against these bacterial strains. Table 4.1 represents the determined MIC values of rhamnolipid against the tested strains. The lowest rhamnolipid MIC value of 0.048 mg/mL was reported against *S. constellatus* and *S. intermedius*. *S. anginosus* and *S. aureus* cultures demonstrated no growth compared to their untreated control cultures at rhamnolipid concentration of 0.097 mg/mL. A high MIC value of 50 mg/mL was reported for rhamnolipid against *E. faecalis* but at 0.097 mg/mL *E. faecalis* growth was inhibited by > 60 %. No growth inhibitory effect of rhamnolipid solutions was monitored against *E. coli* growth when compared to the untreated control (Table 4.1).

4.3.1.2.2 *Lp*-BS minimum inhibitory concentration

The four endodontic pathogens and the two reference strains (*S. aureus* NCTC 8325 and *E. coli* NCTC 10418) were cultured with *Lp*-BS solutions to account for the antimicrobial effect of *Lp*-BS against these bacterial strains. No antimicrobial activity of *Lp*-BS solutions was reported against any of the tested pathogens (Table 4.1). The lack of antimicrobial effect was indicated by no difference in the 24 h-absorbance reading of the *Lp*-BS treated bacterial cultures compared to the untreated growth controls.

Table 4.1 **Minimum inhibitory concentration (MIC) of *Lp*-BS and rhamnolipid** against SAG, *S. aureus, E. faecalis* and *E. coli*. No antimicrobial activity of *Lp*-BS was demonstrated against any of the tested pathogens at the highest concentration tested. MICs of rhamnolipid range from 0.048 mg/mL against *S. constellatus* and *S. intermedius* and 0.097 mg/mL against *S. anginosus* and *S. aureus*. *E. coli* was found resistant to rhamnolipid while *E. faecalis* required an MIC of 50 mg/mL. Data expressed as mean ± SEM.

Bacterial strain	**MIC (mg/mL)**	
	Rhamnolipid	***Lp*-BS**
S. anginosus 670/95	0.097 ± 0.050	ND
S. constellatus S08-07	0.048 ± 0.020	ND
S. intermedius HW13	0.048 ± 0.024	ND
E. faecalis RB17	50.00 ± 0.00	ND
S. aureus NCTC 8325	0.097 ± 0.020	ND
E. coli NCTC 10418	> 50.00 ± 0.00	ND
MIC; minimum inhibitory concentration, ND; not detected.		

4.3.2 Surface coating characterisation

4.3.2.1 Dentine discs

No difference in surface morphology or appearance between the uncoated plain dentine discs and those coated with 50 mg/mL rhamnolipid was noticed when examined by SEM (Figure 4.3a and b respectively). Coating discs with 50 mg/mL *Lp*-BS resulted in clumpy inconsistent layer and uneven distribution over the disc surface (Figure 4.3 c1 and c2).

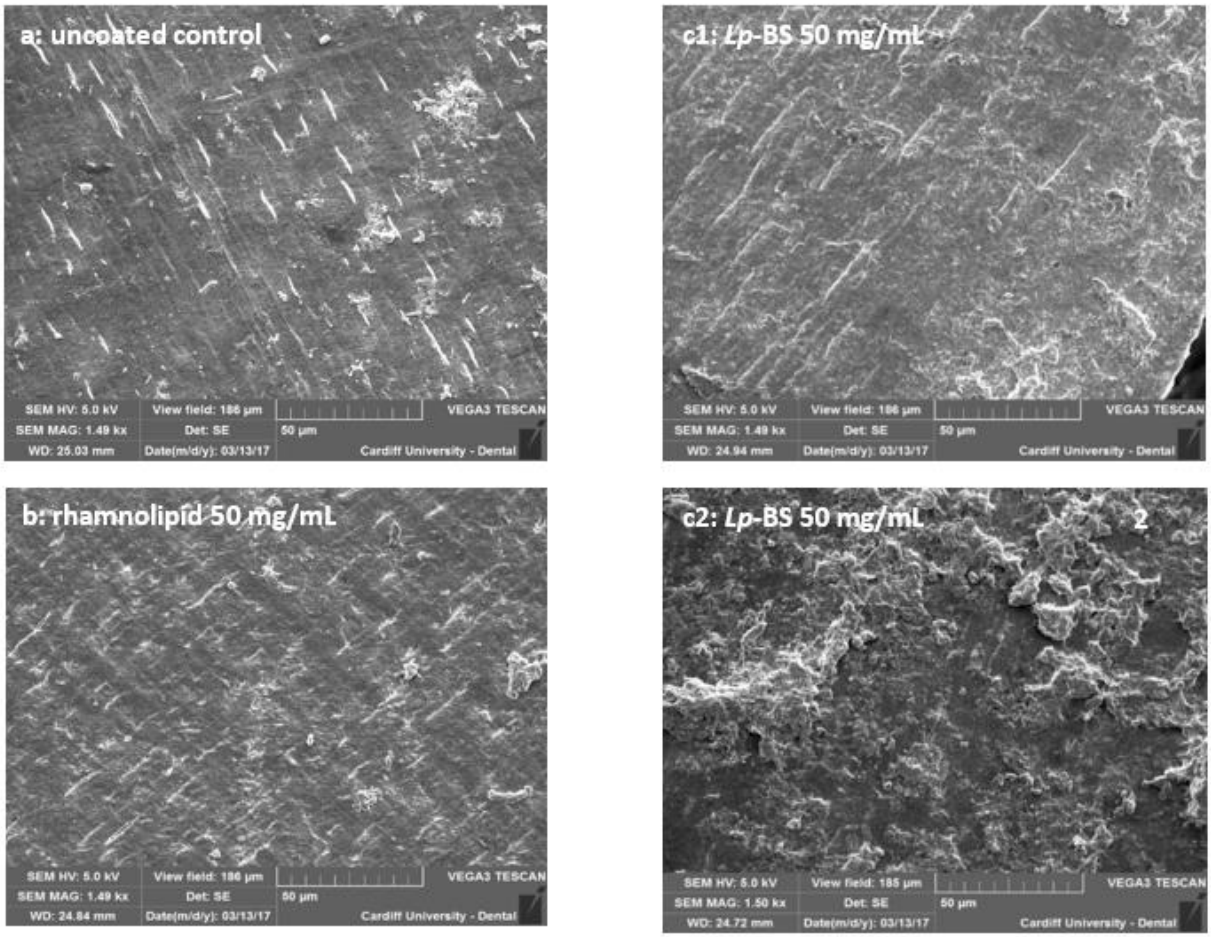


Figure 4.3 **Scanning electron microscopy of dentine discs coated with *Lp*-BS (50 mg/mL) and rhamnolipid (50 mg/mL).** No difference in surface appearance between uncoated control disc **(a)** and disc coated with rhamnolipid was observed **(b)** while disc coated with *Lp*-BS shows clumps or aggregates and uneven distribution (**c1** and **c2**).

4.3.2.2 Acrylic discs

To provide simple, affordable and easy to visualise surface as a coating model, acrylic discs were used. The discs were coated with either of the two biosurfactants (*Lp*-BS or rhamnolipid) at 50 mg/mL as an initial concentration and visualised by SEM. As shown by the SEM images, surface coated with rhamnolipid (Figure 4.4 b1) displayed no difference in appearance to the plain uncoated control (Figure 4.4 a1). Scratching the surface of the coated and uncoated discs as an attempt to reveal any difference in surface texture also resulted in no difference between rhamnolipid treated surface and the uncoated control (Figure 4.4 b2 and a2

respectively). Coupons coated with 50 mg/mL *Lp*-BS showed inconsistent coating of non-homogenous and incomplete layer when examined by SEM (Figure 4.4 c1 and c2).

Following staining with crystal violet, rhamnolipid coating was demonstrated as a light purple colour layer when compared to the uncoated control coupons (Figure 4.5) indicating a successful coating by rhamnolipid. Hence, acrylic coupons were chosen to assess antiadhesive effect of rhamnolipid.

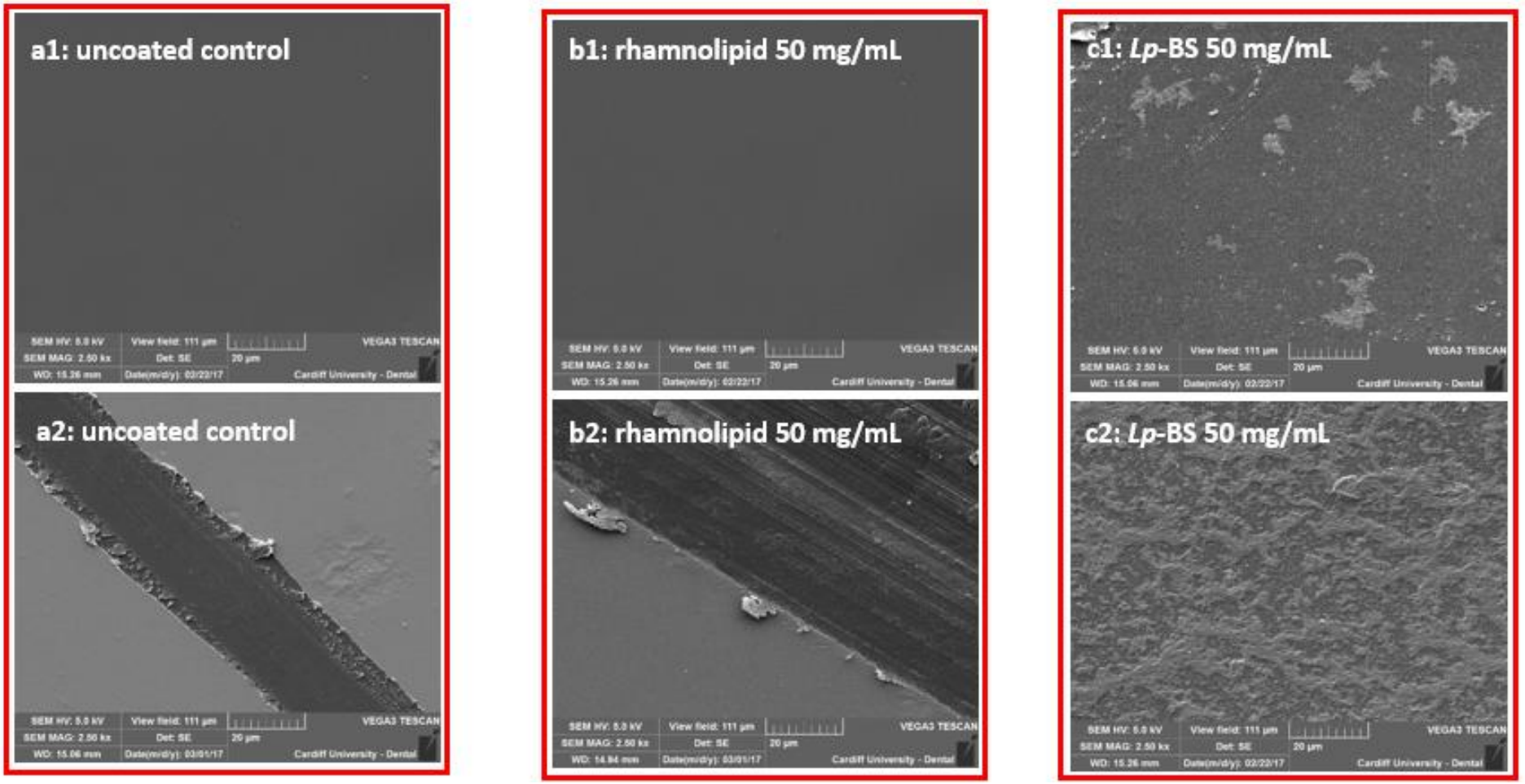


Figure 4.4 **Scanning electron microscopy of acrylic discs coated with *Lp*-BS (50 mg/mL) and rhamnolipid (50 mg/mL).** To study antiadhesive activity of the two biosurfactants, acrylic coupons were used to provide a simple and economic model for the coating which can be visualised under microscope. Discs coated with rhamnolipid **(b)** show no difference in surface texture to the uncoated control **(a)** even after scratching the surface as shown in the lower two images (b2 and a2 respectively). Discs coated with *Lp*-BS show non homogenous coverage over the disc surface (**c1** and **c2**).

Figure 4.5 **Crystal violet staining of acrylic discs coated with rhamnolipid 50 mg/mL**. Crystal violet binds to the mannose moiety of the rhamnolipid molecules. Therefore rhamnolipid coating can be detected with crystal violet. The image is a representative of three independent repeats.

4.3.2.3 Glass slides

SYPRO Ruby fluorescent stain was used to visualise 8-well chamber glass slide coated with 50 mg/mL and 20 mg/mL of *Lp*-BS (Figure 4.6). At the high concentration, the coating was shown to be densely accumulated at the well corners due to sloughing and failure to form continuous coating (Figure 4.6c). However, a lower concentration of 20 mg/mL showed the ability to form a complete homogenous layer of coating over the entire well surface (Figure 4.6b). Therefore an 8-well glass slide surface coated with 20 mg/mL *Lp*-BS was selected for subsequent assessment of the antiadhesive activity of the *L. plantarum* biosurfactant.

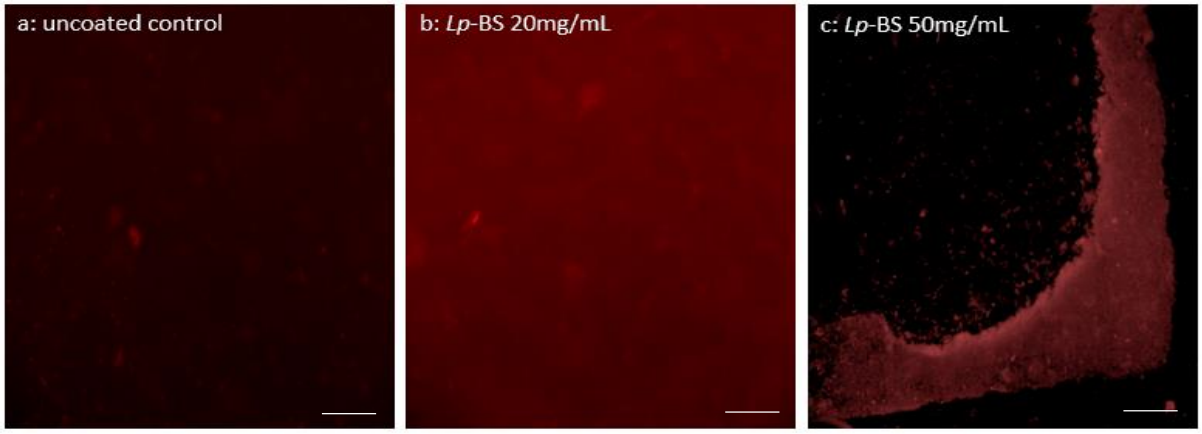


Figure 4.6 **SYPRO Ruby stain for imaging Lp-BS coating of 8-well chamber glass slide using fluorescent microscopy. a-** uncoated control glass slide. **b-** at 20 mg/mL, continuous layer of *Lp*-BS is shown over the whole well surface compared to the control un-coated well (a). **c-** at 50 mg/mL *Lp*-BS, non-continuous coating layer is shown with accumulation at the corners only. 20x magnification was used. Scale bar represents 10 µm.

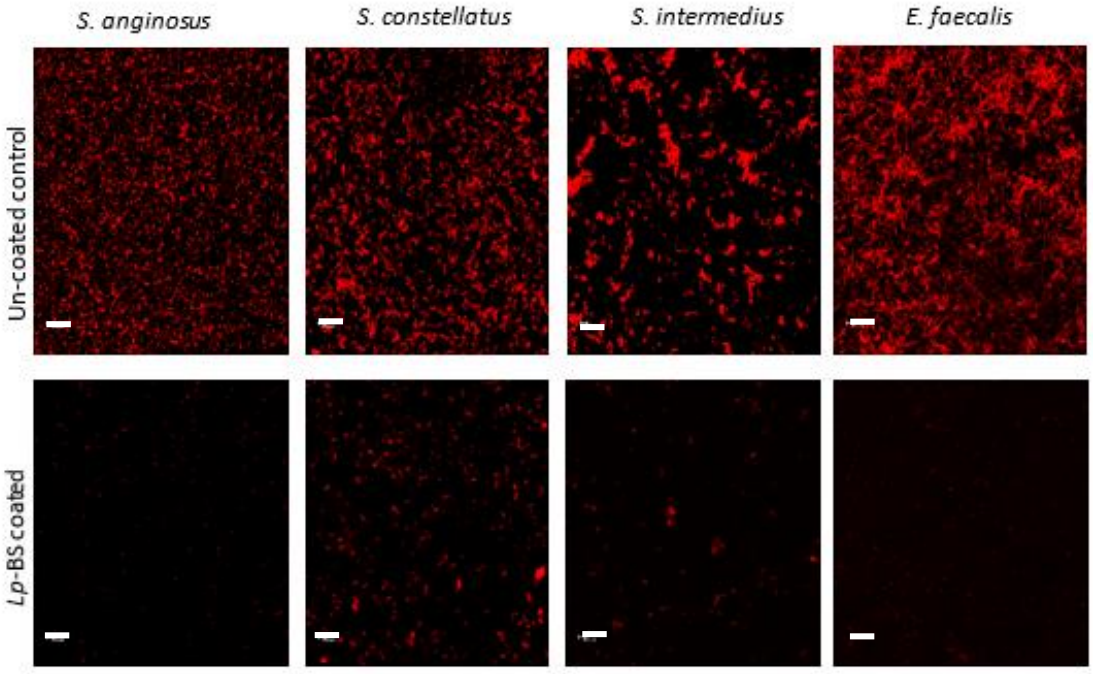
4.3.3 Antiadhesive effect

4.3.3.1 Antiadhesive effect of *Lp*-BS pre-coating of glass slides

4.3.3.1.1 Confocal laser scanning electron microscopy (CLSM)

The antiadhesive activity of the crude *Lp*-BS (20 mg/mL in PBS) was investigated at the bacterial adhesion phase (2 h). Figure 4.7a demonstrates a representation of three independent experimental repeats of the confocal imaging of fixed samples. Pre-treatment of the glass slides with 20 mg/mL *Lp*-BS resulted in a significant ($p < 0.001$) reduction of SAG and *E. faecalis* attachment to the surface when compared to the untreated glass slides as measured by the $\log_{10}$ bacterial covered surface (μm^2) / field (Figure 4.7b).

a-



b-

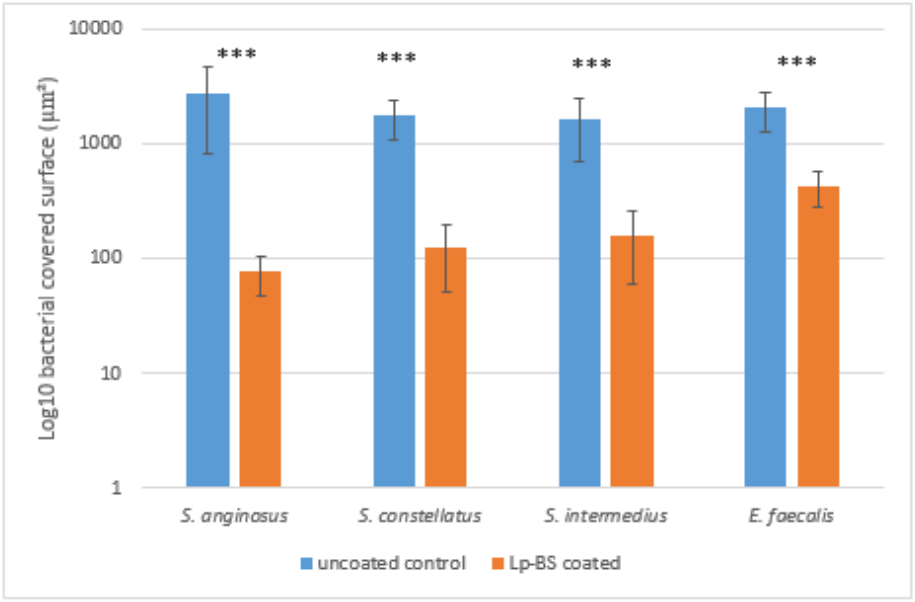


Figure 4.7 **Attachment of SAG and *E. faecalis* to 20 mg/mL *Lp*-BS-coated 8 well chamber glass slide** and to un-coated 8 well chamber glass slide; **a-** surfaces visualised by confocal laser scanning microscopy after fixation and staining with propidium iodide dye using a 40x objectives. Scale bars represent 10 µm, **b-** statistically analysed graph of the bacterial covered surface area. *** $p < 0.001$ using unpaired t-test and error bars represent SEM.

4.3.3.1.2 Recovery of viable bacterial cells

The antiadhesive activity of the crude *Lp*-BS (20 and 10 mg/mL) was further analysed by plating and enumeration of the recovered viable cells after attachment for 2 h to coated and uncoated glass slide surface (Figure 4.8). At 20 mg/mL, *S. anginosus* attachment was significantly reduced ($p < 0.01$) by the effect of biosurfactant coating when compared to the untreated control. Similarly, *S. constellatus* and *S. intermedius* adherence potential to BS-coated surface was found to be significantly lower than that of the plain uncoated surface ($p < 0.05$). *Enterococcus* attachment to the *Lp*-BS-coated surface was also significantly reduced ($p < 0.01$) as demonstrated by lower numbers of viable bacteria recovered. Coating the glass surface with the lower concentration of *Lp*-BS (10 mg/mL) did not significantly reduced SAG and *E. faecalis* adhesion to the coated surface when compared to the uncoated control indicating a concentration-dependent antiadhesive activity of the crude biosurfactant.

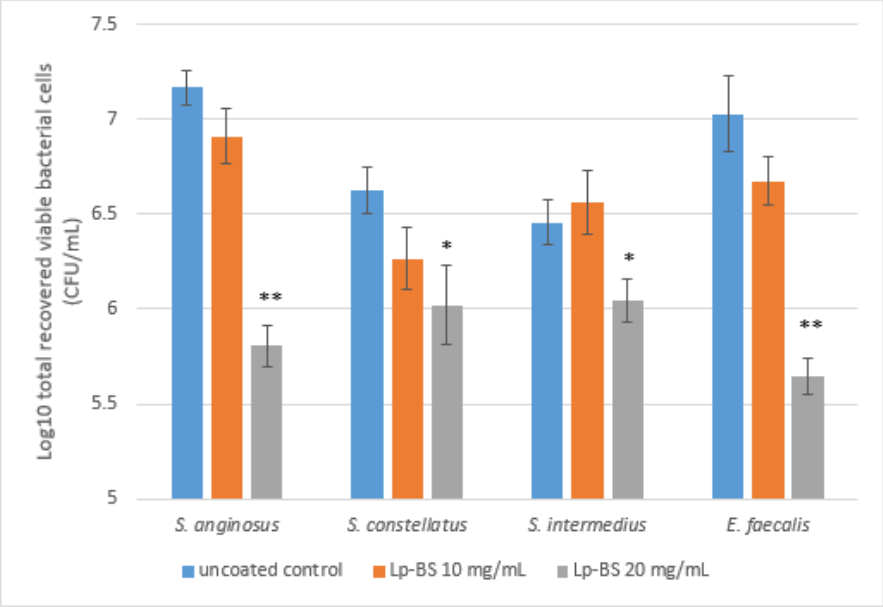


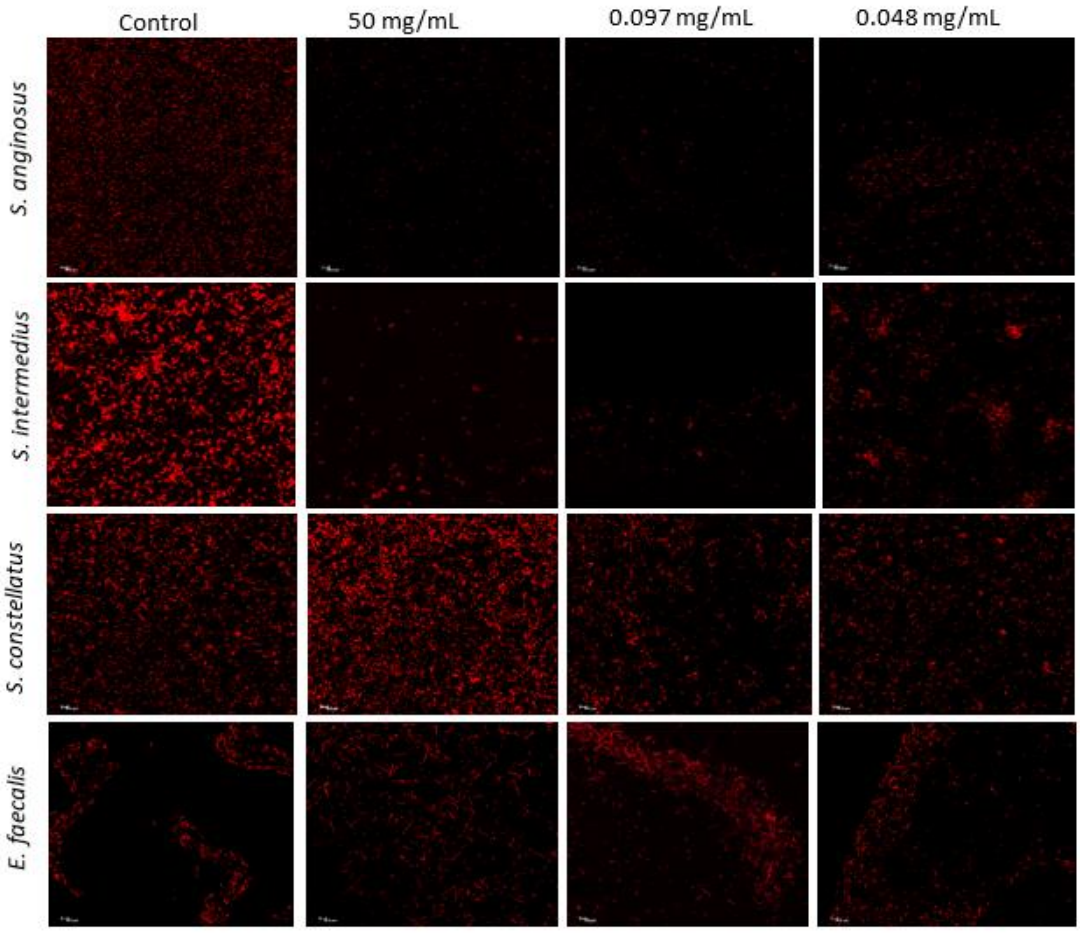
Figure 4.8 **Bacterial cell recovery of SAG and *E. faecalis***. 8 well chamber glass slides were coated with 10 and 20 mg/mL of *Lp*-BS and bacterial attachment were evaluated by recovery of viable cells and enumeration. Error bars represent SEM; ** $p < 0.005$, * $p < 0.05$ using Dunnett's one way ANOVA to the corresponding untreated control.

4.3.3.2 Antiadhesive effect of rhamnolipid

4.3.3.2.1 Confocal laser scanning electron microscopy (CLSM)

Figure 4.9a represents the confocal images of fixed uncoated acrylic coupon controls and coupons pre-coated with different concentrations of rhamnolipid and co-cultured with either member of SAG or *E. faecalis* for 2 h. Figure 4.9b is a statistically analysed graph of the covered surface area of the treated vs. untreated control. *S. anginosus* attachment to discs pre-coated with 50, 0.097 or 0.048 mg/mL rhamnolipid solutions was found to be significantly reduced ($p < 0.001$) compared to the uncoated control coupons. *S. intermedius* adhesion to discs coated with 50 and 0.097 mg/mL was also significantly lowered when compared to the uncoated control ($p < 0.05$) while coating with the lower concentration of 0.048 mg/mL resulted in no significant decrease in attachment ($p > 0.05$). Conversely, no difference ($p > 0.05$) in *E. faecalis* and *S. constellatus* attachment to coated or uncoated coupons was reported at any of the three tested rhamnolipid concentrations.

a-



b-

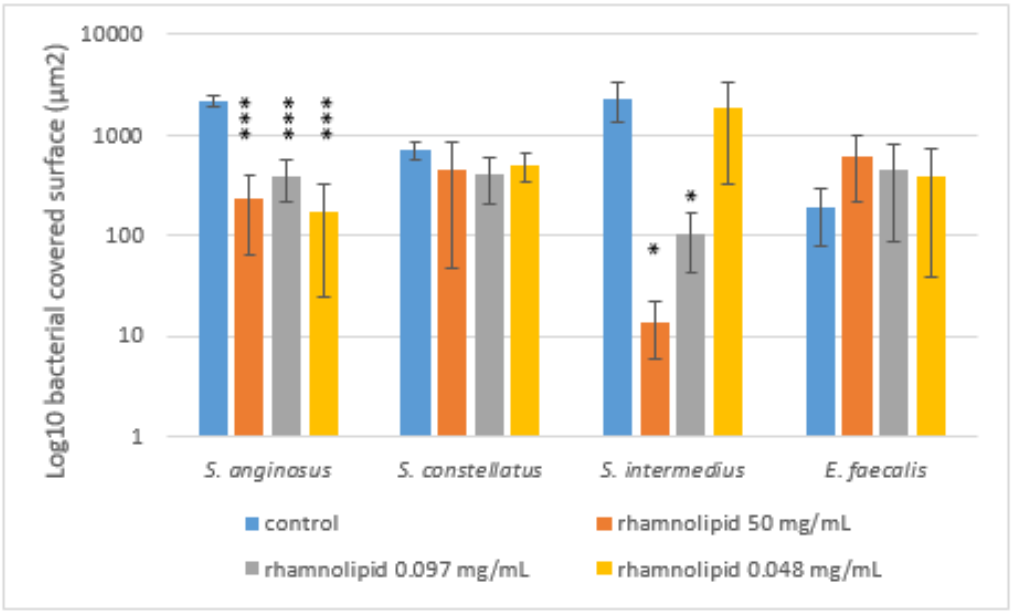


Figure 4.9 **Attachment of SAG and *E. faecalis* to rhamnolipid-coated acrylic discs** (50 mg/mL, 0.097 mg/mL and 0.048 mg/mL) and un-coated acrylic discs. **a-** visualised by confocal laser scanning microscopy after fixation and staining with propidium iodide dye using a 40x objectives. Scale bars represent 10 µm. **b-** statistically analysed graph of the bacterial covered surface area. Error bars represent SEM; *, $p < 0.05$; ***, $p < 0.001$ using Dunnett's one way ANOVA comparing each of the treated group to the untreated control.

4.3.3.2.2 Recovery of viable bacterial cells

Enumeration of the viable cells detached from acrylic coupons coated with rhamnolipid (50, 0.097 and 0.048 mg/mL) revealed a significant reduction ($p < 0.01$) in viability (CFU/mL) of the recovered *S. anginosus* compared to untreated control (Figure 4.10) at the three concentrations tested. *S. intermedius* bacterial cells recovered from discs precoated with rhamnolipid 50 mg/mL failed to be cultured on FAA plates (CFU/mL = 0.0) while the $\log_{10}$ of the bacterial cells recovered from discs precoated with 0.097 and 0.048 mg/mL was significantly ($p < 0.001$) lower than the uncoated control discs (Figure 4.10). *S. constellatus* did not show a significant reduction in attachment to discs coated with 0.097 and 0.048 mg/mL ($p > 0.05$) while no viable cells were recovered from discs coated with the high concentration of 50 mg/mL rhamnolipid. Enumeration of the plated *E. faecalis* bacterial cells recovered from discs coated with rhamnolipid (50, 0.097 and 0.048 mg/mL) and the uncoated control discs showed no significant difference ($p > 0.05$) (Figure 4.10).

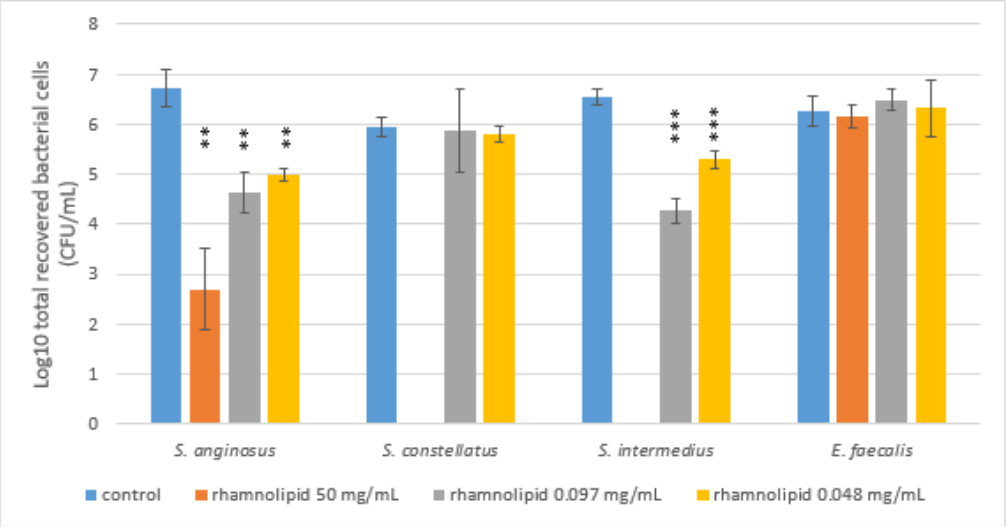


Figure 4.10 **Bacterial cell recovery of SAG and *E. faecalis* from rhamnolipid coated** and uncoated acrylic coupons. Acrylic coupons were coated with 50, 0.097 and 0.048 mg/mL of rhamnolipid solutions and bacterial attachment were evaluated by recovery and enumeration of viable cells. Error bars represent SEM; **, $p < 0.01$; ***, $p < 0.001$ using Dunnett's one way ANOVA comparing each of the treated group to the untreated control group.

4.4 Discussion

Downstream processing of biosurfactants, particularly high molecular weight ones, is probably a very expensive and time consuming process. The production of high yield with low purifying cost is a critical element for commercialisation of powerful biosurfactants (Smyth *et al.* 2010). Therefore there is a trend towards using biosurfactants of partial, but clinically acceptable, purity (Piljac 2012). Accordingly, *Lp*-BS was tested for its antimicrobial/antiadhesive activity in its crude form at this stage of partial purification. Although no antimicrobial effect was observed, surface coating with *Lp*-BS was found to have significant activity in reducing SAG and *E. faecalis* attachment to artificial surfaces. The results obtained herein open future prospects for possible application of this biosurfactant to reduce or inhibit the adhesion of pathogenic microorganisms in several biomedical dental applications for example in prophylaxis and treatment of caries and pulpitis.

Rhamnolipid, a commercially available *P. aeruginosa* derived biosurfactant of 90 % purity was also included in the study to examine its antimicrobial and antiadhesive effect against the above mentioned endodontic pathogens. Rhamnolipid demonstrated a significant antimicrobial activity with MIC values of 50 mg/mL against *E. faecalis,* 0.097 mg/mL against *S. anginosus*, and 0.048 mg/mL for *S. constellatus* and *S. intermedius*. As an antiadhesive agent, rhamnolipid showed the potential to significantly reduce the attachment of *S. anginosus* and *S. intermedius* to *in-vitro* acrylic surfaces whilst *E. faecalis* and *S. constellatus* were not affected by rhamnolipid coating.

Several publications explain the importance of biosurfactants as antimicrobial/antiadhesive agents against various infectious bacteria (Gudiña *et al.* 2010; Rufino *et al.* 2011; Gomaa 2013; Sambanthamoorthy *et al.* 2014; Ndlovu *et al.* 2017; Garg and Chatterjee 2018). However, no previous studies have been published regarding *L. plantarum* NCIMB 8826 biosurfactant antimicrobial/antiadhesive activity against the endodontic infective bacteria SAG and *E. faecalis*. Therefore, experiments were performed to determine whether *Lp*-BS demonstrated any antimicrobial activity against these pathogens. It has been reported that several biosurfactants possess variable degrees of antimicrobial activity including lactic acid bacteria biosurfactants (Banat *et al.* 2000; Rodrigues *et al.* 2004; Moryl *et al.* 2015; Yan *et al.* 2019). However, not all biosurfactants have been documented to exert antimicrobial activity in all cases (Walencka *et al.* 2008; Ciandrini *et al.* 2016; Ndlovu *et al.* 2017). Supporting the current

findings, a recent study by Yan and colleagues (Yan *et al.* 2019) reported a lack of antimicrobial activity of crude cell-bound *L. plantarum* biosurfactant against *S. aureus* at 100 mg/mL. The biosurfactant was extracted by the same method employed herein for *Lp*-BS but no details of the extracted biosurfactant chemical structure were provided by the authors. Conversely, two *L. plantarum* strains isolated from Romanian traditional fermented food products were identified and shown to produce biosurfactants with antimicrobial activity against a number of food-borne contaminants (Cornea *et al.* 2016). Microbial biosurfactants encompass a wide spectrum of chemically diverse compounds such as glycolipids, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids and neutral lipids. Therefore, it is reasonable to expect a diversity of their biological properties including antimicrobial effect against the different pathogenic strains (Santos *et al.* 2016). For instance, *L. plantarum* CFR 2194 was shown to produce a glycoprotein cell-bound biosurfactant with significant antimicrobial activity (Madhu and Prapulla 2014). As revealed from the FT-IR spectrum, this glycoprotein biosurfactant shared the same chemical functional groups with *Lp*-BS. However their FT-IR fingerprint regions are not the same indicating they are glycoproteins of different chemical structures. Similar findings have been reported for *L. agilis*; its cell-bound glycoprotein biosurfactant exhibited considerable antimicrobial activity but shared different chemical structures (as indicated by non-identical FT-IR fingerprint region) (Gudiña *et al.* 2015) to that of *Lp*-BS which lacks antimicrobial activity. There would appear to be a degree of strain-specificity for activity, in that some probiotics affect only some strains of a given species. Therefore it is likely that each strain needs to be verified individually for the health benefit in demand (De Vrese and Schrezenmeir 2008; Rastogi *et al.* 2011).

The antimicrobial activity of the glycolipid, rhamnolipids, has been reported against a wide range of pathogens with significantly variable MIC values (Table 4.2) (Haba *et al.* 2003; Benincasa *et al.* 2004; Magalhães and Nitschke 2013; de Freitas Ferreira *et al.* 2018). However, to the best of our knowledge, the activity against SAG endodontic pathogens has not been studied. The commercial 90 % pure rhamnolipid tested herein exhibited a significant antimicrobial effect particularly against SAG, but inhibited 60 % of *E. faecalis* at the MIC required to inhibit *S. anginosus* (0.097 mg/mL). It has been reported that rhamnolipids act on the cell membrane and the variability of its effect on bacteria may be related to the difference in lipid composition of the cells (Verheul *et al.* 1997; Sotirova *et al.* 2008). Moreover, the

glycolipid rhamnolipids can exist in several isoforms and analogues resulting in significant structural heterogeneity (Benincasa *et al.* 2004). Such structural diversity might be responsible for the variability in reported antimicrobial activity of the rhamnolipids. Strain-specificity is an additional consideration; there is evidence that rhamnolipids produced from different strains have different structures which in turn influence their antimicrobial activity (Déziel *et al.* 1999; HoŠková *et al.* 2013). When compared to the SAG members, *E. faecalis* was found to be less susceptible to the rhamnolipid inhibitory effect (MIC = 0.097 and 50 mg/mL for *S. anginosus* and *E. faecalis* respectively). The ability of *Enterococcus* species to resist many clinically useful antibiotics has been reported as a growing issue in contemporary medicine (Hollenbeck and Rice 2012) and in the context of endodontic infection (John *et al.* 2015). Studies have reported a wide range of MIC values of rhamnolipid against the different *E. faecalis* strains tested. For instance, rhamnolipid extracted from *P. aeruginosa* MR01 did not show antimicrobial activity against clinical and reference *E. faecalis* strains at 512 µg/mL whilst another stain (*P. aeruginosa* MASH1) produced rhamnolipid with antimicrobial activity against the two *E. faecalis* strains at lower concentration (MIC = 64 µg/mL) (Lotfabad *et al.* 2012). Kristoffersen and his group (Kristoffersen *et al.* 2018) extracted rhamnolipid from a strain of the *P. fluorescence* Group which could inhibit > 50 % of *E. faecalis* growth at 50 µg/mL. Another study revealed that rhamnolipid extracted from *P. aeruginosa* LBI demonstrated a much lower MIC value of 4 µg/mL against *E. faecalis* (Benincasa *et al.* 2004) than that stated earlier in the previous example.

Table 4.2 **Review of a number of publications reporting MICs of rhamnolipid of different sources against a range of microorganisms**.

Bacterial strain	Method used	MIC value	Rhamnolipid origin	Reference
S. aureus ATCC 2921	micro-broth dilution	128 μg/mL	*P. aeruginosa* MR01	(Shahcheraghi *et al.* 2013)
	micro-broth dilution	512 μg/mL	*P. aeruginosa* MASH1	(Shahcheraghi *et al.* 2013)
E. faecalis ATCC 29212	micro-broth dilution	>512 μg/mL	*P. aeruginosa* MR01	(Shahcheraghi *et al.* 2013)
	micro-broth dilution	64 μg/mL	*P. aeruginosa* MASH1	(Shahcheraghi *et al.* 2013)
E. faecalis (Clinical sample)	micro-broth dilution	>512 μg/mL	*P. aeruginosa* MR01	(Shahcheraghi *et al.* 2013)
	micro-broth dilution	64 μg/mL	*P. aeruginosa* MASH1	(Shahcheraghi *et al.* 2013)
E. coli (Clinical sample)	micro-broth dilution	>512 μg/mL	*P. aeruginosa* MR01	(Shahcheraghi *et al.* 2013)
	micro-broth dilution	>512 μg/mL	*P. aeruginosa* MASH1	(Shahcheraghi *et al.* 2013)
E. coli ATCC 25922	micro-broth dilution	>512 μg/mL	*P. aeruginosa* MR01	(Shahcheraghi *et al.* 2013)
	micro-broth dilution	>512 μg/mL	*P. aeruginosa* MASH1	(Shahcheraghi *et al.* 2013)
S. aureus	Disc diffusion	17 mm at 10 μg/mL	*P. flourescences*	(Govindammal and Parthasarathi 2013)
E. coli	Disc diffusion	10 mm at 10 μg/mL	*P. flourescences*	(Govindammal and Parthasarathi 2013)
S. aureus ATCC 6538	micro-broth dilution	32 μg/mL	*P. aeruginosa* 47T2 NCIB 40044	(Haba *et al.* 2003)
32 strains of *Listeria monocytogenes*	micro-broth dilution	78.1-2500 μg/mL	-	(Magalhães and Nitschke 2013)
Burkholderia cepacia	micro-broth dilution	1000 g/mL	*P. aeruginosa* BTN 1	(Tedesco *et al.* 2016)
E. coli	micro-broth dilution	32 μg/mL	*P. aeruginosa* AT10	(Abalos *et al.* 2001)
S. aureus	micro-broth dilution	128 μg/mL	*P. aeruginosa* AT10	(Abalos *et al.* 2001)
S. faecalis	micro-broth dilution	64 μg/mL	*P. aeruginosa* AT10	(Abalos *et al.* 2001)
C. albicans	micro-broth dilution	>256 μg/mL	*P. aeruginosa* AT10	(Abalos *et al.* 2001)
S. anginosus 670/95	micro-broth dilution	0.097 mg/mL	*P. aeruginosa* (commercial)	Current study
S. constellatus S08-07	micro-broth dilution	0.048 mg/mL	*P. aeruginosa* (commercial)	Current study
S. intermedius HW13	micro-broth dilution	0.048 mg/mL	*P. aeruginosa* (commercial)	Current study
E. faecalis RB17	micro-broth dilution	50 mg/mL	*P. aeruginosa* (commercial)	Current study
S. aureus NCTC 8325	micro-broth dilution	0.097 mg/mL	*P. aeruginosa* (commercial)	Current study
E. coli NCTC 10418	micro-broth dilution	> 50 mg/mL	*P. aeruginosa* (commercial)	Current study

In addition to their antimicrobial activity, biosurfactants have been widely reported to effectively impede bacterial attachment and colonisation. Coating surfaces with biosurfactants has shown promise as an alternative non-pharmacological anti-infective therapy particularly in the context of increasingly antibiotic resistant pathogens (Gudiña *et al.* 2010; Rufino *et al.* 2011; Gomaa 2013; Satpute *et al.* 2018; Yan *et al.* 2019). It has been reported that *Lactobacillus* spp. biosurfactants, rich in unspecified proteins, possess strong binding affinity to different surfaces (Velraeds *et al.* 1996; Velraeds *et al.* 1996; Satpute *et al.* 2016). However, there is a lack of information regarding the biosurfactants' surface coating characterisation. In the present study, and for the first time, a fluorescent staining with SYPRO Ruby stain was found to be efficient in characterising surface coating with *Lp*-BS. Crystal violet staining was found successful in assessing rhamnolipid coating. Crystal violet staining has been used mainly as an indirect approach for assessing the antiadhesive efficacy of biosurfactants coating (Janek *et al.* 2012) by measuring the amount of bacteria attach to the coated surface. This stain binds to the sugar moiety of bacteria cell wall and biosurfactant coating and therefore might give incorrect reading. Therefore, fluorescent microscopy and viable bacterial cell count were employed herein to assess the antiadhesive activity of *Lp*-BS and rhamnolipid. *Lp*-BS demonstrated a significant antiadhesive activity against SAG and *E. faecalis* attachment to glass surface at 20 mg/mL as revealed by the confocal fluorescent images and the recovered viable bacterial cell counts but not at 10 mg/mL as evident from the recovered viable cell count indicating a concentration-dependent effect. A number of researchers have documented the effectiveness of cell-associated lactobacilli proteinaceous biosurfactants as antiadhesive agents to impede bacterial surface colonisation (Velraeds *et al.* 1996; Velraeds *et al.* 1998; Satpute *et al.* 2016). For instance, *L. plantarum* CFR 2194 glycoprotein biosurfactant was found to effectively inhibit the *in-vitro* attachment of food-borne pathogens (*Escherichia coli* ATCC 31705, *E. coli* MTCC 108, *Salmonella typhi*, *Yersinia enterocolitica* MTCC 859 and *Staphylococcus aureus* F 722) to 96-well ELISA plates via crystal violet method (Madhu and Prapulla 2014). Cell-bound biosurfactant of *L. plantarum* 27172 inhibited *S. aureus* CMCC 26003 adhesion by 50 % at 50 mg/mL with no antimicrobial activity demonstrated at this concentration (Yan *et al.* 2019). Although little information about the mechanism of such antiadhesiveness has been provided by these studies, it is possible that biosurfactants change surface properties such as hydrophobicity making the surface more averse to bacterial attachment (Fischer 1996; Hua *et al.* 2003; Pacwa-Płociniczak *et al.* 2011).

Interestingly, biosurfactants have been proposed to function as antiadhesive agents via manipulating gene expression of different virulent bacterial genes. It has been shown that *Lactobacillus*-derived cell-bound biosurfactant caused down regulation of essential dental-adhesion genes; glucosyltransferases and fructosyltransferase (gtfB, gtfC and ftf) in *S. mutans* strains, a major cariogenic pathogen, and therefore hindered its biofilm formation potential (Tahmourespour *et al.* 2011a; Tahmourespour *et al* 2011b; Salehi *et al.* 2014). In addition, a rhamnolipid-like biosurfactant of *S. mitis* was shown to reduce *S. mutans* adherence to glass surface by the dynamic shear forces that occur in the oral cavity (Van Hoogmoed *et al.* 2000). The identification of ECM-binding proteins in the biosurfactant mixtures has been shown to be involved in the antiadhesion potential of the biosurfactants. These binding proteins may compete with pathogenic bacteria for the protein binding site and hence interfere with their attachment. A 29 KDa protein (p29) possessing antiadhesive activity against a well-known virulent uropathogen, *E. faecalis* 1131 was successfully purified from *L. fermentum* RC-14 biosurfactant mixture. This protein showed 100 % identity to a 29 KDa collagen-binding protein previously isolated from *L. reuteri* NCIB 11951 (Heinemann *et al.* 2000). Later, the same team successfully identified collagen-binding proteins from *L. fermentum* RC-14, *L. rhamnosus* GR-1 and *L. casei* Shirota biosurfactant mixtures using surface-enhanced laser desorption/ionisation (SELDI)-time of flight mass spectrometry as a rapid mean to characterise ECM-binding proteins in the complex but clinically significant biosurfactant mixtures (Howard *et al.* 2000). In the *Lp*-BS mixture, three ECM-binding proteins (elongation factor Tu, enolase 1 and glyceraldehyde 3-phosphate dehydrogenase) have been identified (see Chapter 3). These proteins have not been previously recognised in biosurfactant compounds with antiadhesive activity. However, their involvement in protein-binding (Dhanani and Bagchi 2013, Castaldo *et al.* 2009; Kainulainen and Korhonen 2014; Salzillo *et al.* 2015; Kinoshita *et al.* 2008) could explain the observed significant antiadhesive effect of *Lp*-BS. Nonetheless, further investigations are required to confirm the relationship between the antiadhesive effect of *Lp*-BS and these identified adhesion proteins.

The glycolipid rhamnolipid demonstrated a selective potential to reduce the adhesion of *S. anginosus* and *S. intermedius* but not *S. constellatus* and *E. faecalis* which might be due to a higher adhesion tendency of the two latter strains to the acrylic surface. At 50 mg/mL, a significant lower number of viable cells of *S. anginosus* and no cells of *S. constellatus* and *S.*

intermedius were recovered from the coated coupons. This might be due to the antimicrobial activity of rhamnolipid coating layer on attached SAG bacterial cells.

4.5 Conclusion

This study represents the first description of the antimicrobial/antiadhesive activity of *L. plantarum* NCIMB 8826 biosurfactant (*Lp*-BS) and rhamnolipid against the endodontic pathogens, SAG and *E. faecalis*. Moreover, *Lp*-BS biosurfactant coating was successfully detected, for the first time by means of conventional stains. The antiadhesive propensity reported for *Lp*-BS and the antimicrobial activity reported for rhamnolipid seems to be particularly promising in laying the foundation for a new therapies in the field of endodontics which is particularly welcome, given the growing problem of antimicrobial resistance. However, further investigations are required to assess the cytotoxic effect of these two substances to viable pulp cells and tissues.

5 Chapter 5 *Ex-vivo* application of *Lp*-BS and rhamnolipid

5.1 Introduction

The preceding chapters (Chapter 3 and Chapter 4), characterised and assessed for the *in-vitro* antimicrobial/antiadhesive activity of the two biosurfactants *Lp*-BS and rhamnolipid. However, to demonstrate further translational potential before clinical use, the two biosurfactants were required to be tested on mammalian tissue. Pulpal tissue healing and formation of reparative dentine is the goal of treating exposed pulp in order to preserve pulp vitality and health. The recently introduced concept of vital pulp therapy (VPT) encompasses irritant debridement and application of a protective material over the pulp (Aguilar and Linsuwanont 2011). To minimise bacterial leakage, this procedure must be followed by an overlying tight-sealed restoration. Although successful outcomes of VPT procedures in vital teeth with cariously exposed pulp has been documented in several studies (Mejare and Cvek 1993; Calişkan 1995; Matsuo *et al.* 1996; Demarco *et al.* 2005), another set of studies (Amini and Parirokh 2008; Dammaschke *et al.* 2010a; Akhlaghi and Khademi 2015; Hargreaves and Berman 2015) demonstrated a decreased success rate with time. These unfavourable outcomes were shown to be mainly due to infection caused by either residual bacteria or new ones leaked through the restoration margins (Neelakantan *et al.* 2012; Akhlaghi and Khademi 2015). This observation suggests the need for the use of pulp-capping agents with antimicrobial activity underneath permanent restorations (Neelakantan *et al.* 2012). The currently available pulp capping materials such as calcium hydroxide, polyantibiotic paste, light-cured fluoride-releasing hydroxyapatite-based liner and mineral trioxide aggregates have been shown to exert antimicrobial activity to a varying extent (Lu *et al.* 2008; Reston and de Souza Costa 2009; Pereira-Cenci *et al.* 2013; Shivakumar *et al.* 2009) although these cavity sterilising agents may induce pulp damage themselves (Neelakantan *et al.* 2012). Biosurfactants, microbial-derived compounds with surface activity containing both hydrophilic and hydrophobic moieties (Costa *et al.* 2011) are the focus of recent interests due to their biodegradable and environment friendly nature, biocompatibility, stability in extreme conditions of temperature, pH and salinity (Banat *et al.* 2000). Alongside these physicochemical properties, biosurfactants have potential applications in medical and pharmaceutical fields for their microbial growth inhibitory and antiadhesive properties (Rodrigues *et al.* 2007). In addition, biosurfactants

have been shown to exert healing and immune-modulatory effects on tissues (Stipcevic *et al.* 2013; Sana *et al.* 2017; Sen *et al.* 2019).

Therefore, the introduction of such multifunctional molecules as a novel endodontic material can potentially provide a protection to the pulp not only through antimicrobial activity but also by preventing bacterial colonisation of the pulp ECM. *Lp*-BS with its significant SAG-antiadhesive activity obtained herein, as a probiotic-derived biosurfactant, might offer the advantage of being non-toxic to the tissue. Although derived from *Pseudomonas sp*., rhamnolipids are accepted for *in-vivo* safety according to tests results on animals (Sekhon Randhawa and Rahman 2014). Owing to their amphiphilic nature and diverse chemical structure, rhamnolipids have found a wide range of applicability in the fields of environment bioremediation, foods, cosmetics, and biomedicines (Abdel-Mawgoud *et al.* 2010). The relatively cost effective commercial rhamnolipid tested herein has been shown to effectively inhibit SAG members' growth.

A previously developed *ex-vivo* rodent dental model (Roberts *et al.* 2013), provides a way to study the cellular and molecular response to treatment with new endodontic antimicrobial agents. Hence, the aim of this chapter was to investigate the *ex-vivo* effect of *Lp*-BS and rhamnolipid as antimicrobial and antiadhesive agents. In addition, the viability of the pulp cells and tissues in response to such treatment was assessed. As bacterial derived products, the immunogenicity of the two biosurfactants was also evaluated.

5.2 Materials and methods

5.2.1 Rat dental pulp extraction

Rat dental pulp was extracted according to a previously developed protocol (Harrington *et al.* 2014). Male Wistar rats (28-day-old) were sacrificed by a qualified technician at the Joint Biological Services Unit, Cardiff University for harvesting of tissue under schedule 1 of the UK Animals Scientific Procedures Act, 1986. Four rats were bathed for 30 s in 70 % IMS for sterilisation. Incisions were made along the soft mucosa of the mouth to allow easier access to the teeth, then the connective tissue surrounding incisors was cut using a scalpel and the incisors extracted using forceps. Pulp tissue was obtained by teasing out the contents from the exposed end of the incisors or by dividing the incisor longitudinally to reveal the pulp tissue. All pulp tissue was pooled and kept hydrated in antibiotic treated α-modified Minimum Essential Medium (αMEM) containing ribonucleosides, deoxyribonucleosides, L-glutamine and phenol red, in addition to antibiotics (100 units/ml penicillin G sodium, 0.1 µg/ml streptomycin sulphate and 0.25 µg/ml amphotericin; Invitrogen, Paisley, UK).

5.2.2 Production of a single cell suspension from dental pulp tissues

Rat dental pulp cells were obtained according to a previously developed protocol (Harrington *et al.* 2014). The extracted pulp tissue was quickly shredded using a scalpel and immediately treated with pre-warmed 4 mg/mL collagenase/dispase (Roche Applied Science, UK) at 37 °C for 1 h with periodic shaking. The swift processing minimises potential inflammatory responses by the pulp tissue. The dissociated cells were passed through a 70 µm cell strainer (BD Falcon, UK) and a 5-10 mL medium containing serum were also passed through the strainer to produce a single cell suspension. The cells were centrifuged at 400 *g* for 5 min before the supernatant was discarded and the cell pellet resuspended with fresh medium, then centrifuged again to ensure complete removal of the enzymes. The cells were then resuspended in culture medium.

5.2.3 General culturing medium

Working medium for cell culture consisted of α-modified Minimum Essential Medium (αMEM) containing ribonucleosides, deoxyribonucleosides, L-glutamine and phenol red, in addition to antibiotics (100 units/ml penicillin G sodium, 0.1 µg/ml streptomycin sulphate and 0.25 µg/ml amphotericin; Invitrogen, Paisley, UK) with 20 % heat inactivated foetal bovine serum (FBS) (Invitrogen, UK). The rodent tooth sections were cultured in Dulbecco's Modified

Eagle's Medium (DMEM) with phenol red (Sigma 41965-047) with/without antibiotics (100 units/ml penicillin G sodium, 0.1 µg/ml streptomycin sulphate and 0.25 µg/ml amphotericin; Invitrogen, Paisley, UK) supplemented with 10 % heat inactivated FBS and 0.15 mg/ml vitamin C.

5.2.4 Cell Culture

Cell culture was performed under sterile conditions, using tissue culture plates and flasks (Sarstedt, Leicester, UK). Cells were cultured in 5 % CO_2 in a humidified chamber at 37 °C. Medium was pre-warmed to 37 °C in a water bath, prior to administering to the cells. When the cells were approximately 80 % confluent in T-75 flask, they were washed with 0.1M phosphate buffered saline (PBS, pH 7.4) before being treated with 2 ml of pre-warmed accutase (Accutase® solution. Sigma Aldrich). Incubation was performed at 37 °C until the cells had become detached from the culture vessel (approximately 5 min). Accutase was subsequently inactivated by the addition of serum-containing in the culture medium before centrifugation at 1800 rpm. The resulting pellet was resuspended in the culture medium before cell counting and reseeding as described before.

5.2.5 Freezing and Thawing Cells

Cells were centrifuged, stained with trypan blue and counted (Sigma, UK) and resuspended in 1 mL freezing mix containing 10 % Dimethyl Sulfoxide (DMSO) (Sigma, UK) and 90 % FBS. Freezing mix was added to the cryovials placed in freezing pot at -70°C. After 24 h, cryovials were transferred to liquid nitrogen for long term storage. When cells were required, cells were rapidly thawed by transferring to a 37 °C water bath. The cells were immediately resuspended in 5 mL medium and centrifuged as stated above. Cells were rewashed with medium and expanded in culture.

5.2.6 *Lp*-BS and rhamnolipid cytotoxicity on rat dental pulp cells

The possible toxic effect of the two biosurfactants (*Lp*-BS and rhamnolipid) on extracted rodent dental pulp cells was assessed by trypan blue staining. Trypan blue is taken up by the cells, however, only viable cells are able to subsequently exclude the dye out (Strober 2001). The stain, therefore, is only present in non-viable cells. From confluent culture, 6-well plates were seeded at 4000 cell/cm^2 density and incubated in for 24 h. At the end of incubation, medium was aspirated and cells washed with PBS. Different concentrations of *Lp*-BS (20, 10 and 5 mg/mL) and rhamnolipid (50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195 and 0.097

mg/mL) in αMEM culture medium (Section 5.2.3) were prepared. Two mL of each treatment solution was added to the corresponding wells in triplicate. Medium with 0 mg/mL of either biosurfactants was served as the negative control. The plates were incubated in 5 % CO_2 in a humidified chamber at 37 °C for another 24 h. After 24 h incubation, the medium was removed, cells were washed three times with PBS and incubated with 0.5 mL 0.4 % trypan blue stain for 1 min. The stain was removed and the cells were washed once with PBS. The numbers of blue stained and non-stained cells were immediately counted under light microscopy. Viability greater than 90 % was deemed to be acceptable.

5.2.7 Detection of apoptosis induced by *Lp*-BS and rhamnolipid by TUNEL assay

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick End Labeling (TUNEL) assay has been designed to detect apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis (Kyrylkova *et al.* 2012). The method is based on the ability of TdT to label blunt ends of double-stranded DNA breaks (Kyrylkova *et al.* 2012). From confluent cell culture (Section 5.2.4), cells were seeded at 4000 cell/cm^2 density using eight well chamber glass slide. Chamber slides were incubated in 5 % CO_2 at 37 °C for 24 h. At the end of the incubation period, the medium was discarded and attached cells washed with PBS. Three hundred microliters of rhamnolipid solution of 0.097 and 0.195 mg/mL concentration or *Lp*-BS solution of 5 and 10 mg/mL in αMEM + 20 % FBS were added to the corresponding wells in triplicates. Control wells (negative and positive) were incubated in αMEM + 20 % FBS only. The test and control chamber slides were incubated in 5 % CO_2 at 37 C for 24, 48 and 72 h. At the end of each incubation period, wells were treated according to the manufacturer's protocol (DeadEnd™ Fluorometric TUNEL System, Promega, UK). Briefly, cells were fixed with 4 % formaldehyde in PBS for 25 min at 4 °C. The slide chambers were removed and then washed twice by immersing the slides twice in PBS, 5 min each time. Cells were then permeabilised by immersing the slides in 0.2 % Triton® X-100 in PBS for 5 min to allow penetration of the TUNEL reaction reagents into the cell nucleus followed by washing by immersing the slides twice in PBS, 5 min each time. A positive control was prepared by treating cells, cultured in the medium only with no biosurfactants, with 100 µL (5 unit/mL) DNase (Promega) per well for 5 min at room temperature to induce DNA strand breaks, prior to labelling. One hundred µL of equilibration buffer was added to the cells at room temperature for 5-10 min. While cells were equilibrating, rTdT incubation buffer was

prepared by addition of 45 µL equilibration buffer to 5 µL nucleotide mix and 1 µL rTdT enzyme. Fifty µL of rTdT incubation buffer was added to the cells of the experimental and positive control. Incorporation of biotinylated-dUTP onto the 3' ends of fragmented DNA is facilitated in the reaction containing TdT enzyme. The slides were then covered with plastic coverslips to ensure even distribution of the mixture and incubated for 60 min at 37 °C in a humidified chamber protected from light exposure by wrapping the slide container with aluminium foil. The slides were kept protected from light to the end of the experiment. The reaction was stopped by immersing the slides in 2X SSC for 15 min followed by washing three times in PBS, 5 min each time. To visualise all nuclei, 10 µL Vectashield® with DAPI was added to the wells. Detection of localised green fluorescence of apoptotic cells was performed by imaging the slides under fluorescence microscopy–equipped with fluorescein isothiocyanate (FITC) filter where they appeared green (TUNEL positive). DAPI-stained nuclei were stained blue. The percentage of TUNEL-positive cells was calculated as the number of TUNEL-positive cells divided by the number of cells stained blue.

5.2.8 Tooth slice culture under standard conditions

Tooth slices were prepared as previously described (Roberts *et al.* 2013). Male Wistar rats (28-day-old) were sacrificed under schedule 1 of the UK Animals Scientific Procedures Act, 1986 by a qualified technician at the Joint Biological Services Unit, Cardiff University for harvesting of tissue. Upper and lower incisor teeth were dissected from the freshly sacrificed rats by removing the bone from around the teeth using a sterile scalpel. The teeth were then placed in sterile DMEM (Section 5.2.3) prior to being cut into 2 mm thick transverse sections on a diamond edged rotary saw (TAAB, Berkshire, UK). The blade was sterilised using 70 % ethanol and was kept cool with sterile PBS in a well at the base of the blade. The cut sections were immediately transferred to fresh sterile DMEM + 20 % FBS for no more than 20 min before being cultured in 2 mL of DMEM + 20 % FBS at 37 °C, 5 % CO_2. Storage of the dissected incisors and resulting tooth slices in supplemented DMEM before culture is vital to maintain maximum viability of the cells within the slices. Figure 5.1 demonstrates the orientation of the tooth slice from which the section is cut, relative to its position in the incisor.

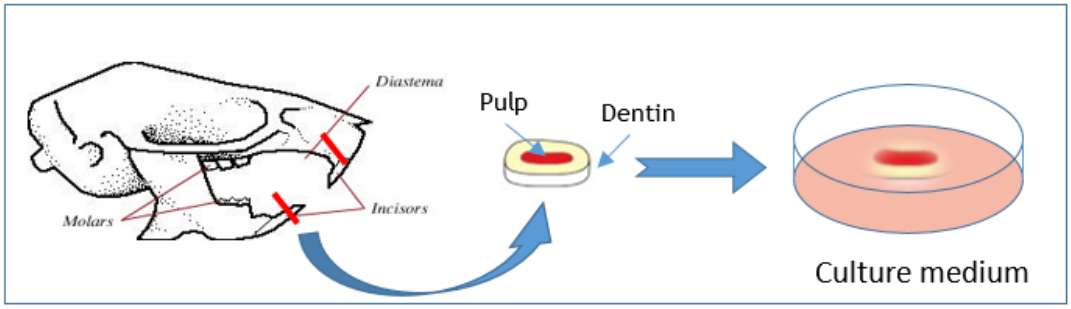


Figure 5.1 **Schematic diagram of rat skull showing position of incisors**. Red areas on schematic represent 2 mm sections that are cut along the length of the incisors. The right-hand side of the diagram shows how the tooth slices appear after being cut and how the tooth slice is orientated in relation to its previous position in the tooth.

5.2.9 Viability of rat pulp tissue follow treatment with *Lp*-BS solutions

5.2.9.1 Incubation of tooth section in *Lp*-BS solutions

Tooth sections were prepared as discussed above (Section 5.2.8) and pre-incubated for 24 h in DMEM + 10 % FBS (Section 5.2.3) at 37 °C, in 5 % CO_2. *Lp*-BS solutions in DMEM + 10 % FBS were prepared by dissolving 10 mg per mL of the medium and then serially 1:1 diluted four times to reach 0.625 mg/mL ending up with *Lp*-BS *of* 10, 5, 2.5, 1.25 and 0.625 mg/mL concentrations. At the end of the 24 h pre-incubation, the medium was discarded and wells were washed with 2 mL PBS. Two mL of *Lp*-BS solutions were added to the corresponding wells in triplicate for each dilution. Negative control tooth sections were incubated in DMEM + 20 % FBS only. The plates then incubated for another 24 h at 37 °C, in 5 % CO_2. Another plate was prepared as above but the tooth sections were treated with *Lp*-BS 10 and 0.625 mg/mL for 48 h at 37 °C, in 5 % CO_2.

5.2.9.2 Histological, fixation, processing and staining methods

5.2.9.2.1 Fixation and demineralisation

Following culture, incisor slices were removed using sterile forceps and transferred to 10 % (v/v) neutral-buffered formalin solution for 24 h for fixation of tissues to occur. The slices

were then transferred to a minimum of 5 mL 10 % (v/v) formic acid for 48 - 72 h with constant agitation to allow for demineralisation.

5.2.9.2.2 Processing of tissue

Tooth slices were transferred to individual biopsy cassettes and processed through a series of graded ethanol on an automatic tissue processor (50 % v/v, 70 % v/v, 95 % v/v, and 100 % v/v ethanol) followed by 100 % v/v xylene for five minutes each. Following processing, the tissues were embedded in paraffin wax using a processing machine (Shandon Pathcentre, Thermo Scientific, Surrey, UK) and aligned so that transverse sections of the slice could be cut.

5.2.9.2.3 Sectioning of tooth slices

Sections of 5 µm thickness were cut from the wax blocks using a Leitz 1400 microtome (Leica, Buckinghamshire, UK). The cut sections were floated on warm water (40 °C) and mounted on glass slides. These were then placed in a slide drying oven (60 °C) before staining to improve adhesion of the section to the slide.

5.2.9.2.4 Staining histological sections

Histological sections were mounted on uncoated microscope slides and placed on an automated tissue stainer. The slides were taken through xylene, alcohol and water washes followed by haemotoxylin and eosin (H&E) stains. They were then taken through a further series of alcohol washes before finally being immersed in xylene. The slides were then removed and a coverslip glued by DPX over the stained section for viewing by an Olympus AX40 light microscope.

5.2.9.2.5 Semi-quantification of cell viability by cell counts

ImageJ software (National Institutes of Health, Maryland USA) was used to count the number of nuclei per pulp on stained histological sections. Images were captured at 20x magnification and five random 50 µm² areas were selected for each section with three sections being studied for each treatment and control group. The blue field was extracted from the images and the moment's threshold method was applied to separate the pulp cells. The watershed function was applied to split adjacent cell nuclei and the number of particles ranging in size from 3 to 100 μm^2 were counted.

5.2.10 RT-qPCR of cytokines

5.2.10.1 RT-qPCR of cytokines of *Lp*-BS treated tooth section

To ensure enough mRNA is extracted from the pulp tissue (Ayre *et al.* 2018), 4 mm thick tooth slices were prepared and cultured as previously described (Section 5.2.8) for 24 h before being transferred to sterile DMEM+10 % FBS as a control or with *Lp*-BS (10 and 0.625 mg/mL prepared in DMEM+10 % FBS) and incubated for another 24 h. After incubation, the tooth slice was transferred to sterile PBS and the pulp removed by flushing the pulpal cavity with PBS using a 0.1 mm needle and syringe. mRNA was extracted using TRIzol® Reagent (ThermoFisher Scientific, Loughborough, UK) followed by DNAse treatment (Promega, Southampton, UK) according to the manufacturers' instructions. Analysis of gene expression was performed in accordance to the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines (Bustin *et al.* 2009). RNA concentrations were determined using a NanoVue Spectrophotometer (GE Healthcare Life Sciences, Buckinghamshire, UK). RNA purity was determined by ensuring the ratio of absorbance at 260/280 nm was above 1.8. RNA was directly stored at -80°C for use in reverse transcription reactions.

Complementary DNA (cDNA) was synthesised by reverse transcription using Promega reagents (Southampton, UK) in a G-Storm GS1 thermocycler (Somerton, UK). One µg of the extracted mRNA was combined with 1 µL random primer in a 15 µL reaction in nuclease free water at 70 °C for 5 min. This suspension was added to 5 µL Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV) reaction buffer, 1.25 µL deoxyribonucleotide triphosphates (10mM stock dNTPSs), 0.6 µL RNasin, 1 µL MMLV enzyme and 2.15 µL nuclease free water and incubated at 37 °C for 1 h. The resultant cDNA was diluted 1:10 in nuclease free water (250 ng cDNA) and stored at -20 °C.

A list of the genes that were examined in this study can be found in table 5.1 with the forward and reverse primers. The primers were designed and validated by Primerdesign Ltd (Primerdesign). Ten µL of PrecisionFAST qPCR SYBR Green MasterMix with low ROX (Primerdesign) was combined with 1 µL resuspended primer mix (Primerdesign) and 4 µL nuclease-free water (Primerdesign, Chandler's Ford, UK) prior to addition of 5 µL cDNA in BrightWhite Real-time PCR FAST 96-well plates (Primerdesign). Plates were covered with adhesive optical seals (Primer Design); and centrifuged at 500 *g* for 5 min. qPCR analysis was

conducted in a QuantStudio™ 6 Flex Real-Time PCR System machine (ThermoFisher Scientific, USA), using QuantStudio™ Real-Time PCR Software (v1.0). Reaction conditions were as follows: one initial denaturation at 95°C for 20s, forty cycles of denaturation for 1s and annealing for 20s. After cycling was completed, melt curve analysis was performed as follows: 95 °C denaturation for 15s, 60 °C dissociation for 1min and 95 °C denaturation for 15s. Relative gene expression was calculated with beta-2-microglobulin (B2M) was used as an internal reference control for data normalisation and negative control samples as the control using the Livak method (Livak and Schmittgen 2001) (i.e. ΔCt method).

5.2.10.2 RT-qPCR of cytokines of rhamnolipid treated tooth section

Four mm thick tooth slices were cultured as previously described (Section 5.2.8) for 24 h before being transferred to sterile DMEM + 10 % FBS as a control or with 0.097 mg/mL rhamnolipid prepared in DMEM + 10 % FBS. The negative control and the treated tooth sections were then incubated for 2, 4, 6, 24, 48 and 72 h at 37 °C in 5 % CO_2. Medium was changed every 24 h. After each incubation time point, tooth sections were treated following the same procedure explained above (Section 5.2.10.1).

Table 5.1 **Primer sequences and product sizes of selected markers for qPCR analysis** used in this study. Note: all the genes used were designed and validated by Primerdesign Ltd, UK. For the reference gene B2M primer sequences refer to Primerdesign Ltd, UK.

Gene	Primer Sequence Forward (F) & Reverse (R)	Product Length (b p)	Annealing Temperature °C
IL-1beta	F:AGCACCTTCTTTTCCTTCATCTT R:CAGACAGCACGAGGCATTTT	145	57.2
IL-6	F:AGAGCAATACTGAAACCCTAGTTC R:GTCCTTAGCCACTCCTTCTGT	133	57.3
TNF-alpha	F:GCTCCCTCTCATCAGTTCCA R:CTCCTCTGCTTGGTGGTTTG	109	56.8
Defensin beta 2 (Defb2)	F:TGCTGGAATTCTAGGACCTCTTC R:GCAACAGTAGAACTTGTATGGGAAA	139	57.6
TGF-beta1	F:CGCAACAACGCAATCTATGAC R:GGACAGCAATGGGGGTTCT	108	57.3
IL-4	F:CACTTTGAACCAGGTCACAGA R:CTCGTTCTCCGTGGTGTTC	94	56
IL-10	F:CTGGACAACATACTGCTGACA R:GGCATCACTTCTACCAGGTAAA	107	56.3
Fas	F:GCACAACAGCCCCCAAGAT R:GCTTTTCTGAGACCCTGGATTAAA	114	57.9
Casp3	F:AGAGTTTGGTGCCACTATGAAT R:CTTGACATTATCGTTCCTATGCTTAA	116	57
Casp8	F:TCCACAGTTGTCTTTACGATATTGC R:GGTCCAAGCACAGGAACTTGA	78	57.7
Bcl2	F:CATGAAATAAAAAGCTGAAAGGAATT R:GGATGAAGGGGTGTCTTTAGTTA	109	56.3

5.2.11 Bacterial growth under modified culture medium (DMEM+10 % BHI)

A previously validated (Roberts *et al.* 2013), modified co-culture medium was used to account for the viability of the study bacterial isolates in this medium. Bacterial suspensions of SAG and *E. faecalis* at 10^2 CFU/mL were prepared by the inoculation of DMEM (Invitrogen) supplemented with 10 % heat-inactivated FBS, 0.15 mg/mL vitamin C and 10 % BHI broth (DMEM-BHI). Bacterial suspensions were also prepared in BHI alone. Two hundred µL of each bacterial isolate suspension (10^2 CFU/mL) in DMEM-BHI and BHI were added to the wells of 96-well plate in triplicate, un-inoculated BHI was served as the negative control. The plate was incubated in a plate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) at 37 °C in 5 % CO_2 and the absorbance (600 nm) was recorded for 24 h. Viable cell count was performed for the first 8 h incubation of the four isolates in DMEM-BHI. Hourly and for 8 h, 50 µL of each bacterial isolate grown in the modified culture medium were plated on FAA plate using spiral platter. The plates were then incubated at 37 °C in 5 % CO_2 for 24 h and the resulting colonies were counted.

5.2.12 *Ex-vivo* rhamnolipid antimicrobial activity

5.2.12.1 Co-culture tooth section with bacteria and rhamnolipid

Two mm tooth sections were prepared as described in section 5.2.8 and pre-incubated for 24 h in supplemented DMEM with antibiotic (Section 5.2.3). Tooth sections were then washed in 2 mL of PBS, transferred into supplemented DMEM without antibiotics and incubated overnight to remove traces of antibiotic. Rhamnolipid solution was prepared at 0.097 mg/mL in serum-free DMEM-BHI with no antibiotic and 1.98 mL of this solution was added to each of the test sections. The same volume of serum-free DMEM-BHI medium with no antibiotic was added to the untreated infection control samples. *S. anginosus* and *S. constellatus* were cultured to the log phase in BHI for 8-12 hours and adjusted to OD (600nm) of 0.08-0.1 equivalent to 10^8 CFU/mL. Twenty µL of this bacterial suspension were added to each of the rhamnolipid treated and the infection control. The tooth sections were then incubated at 37 °C and 5 % CO_2 for 24 h under constant agitation at 60 rpm. At the end of the incubation period, tooth section samples were collected for histology (Section 5.2.12.2) while their culture supernatants were collected for cytokines quantification by ELISA (Section 5.2.13).

5.2.12.2 Tooth slices fixation, processing and sectioning

Tooth sections cultured with the bacterial isolates and rhamnolipid were fixed and processed as described previously in section 5.2.9.2.

5.2.12.3 Histological sections staining with a modified Gram stain

Gram staining of the histological sections was performed using a modified Brown and Brenn method (Ayre *et al.* 2018). Paraffin-embedded tooth slices were cut using a microtome into 5 µm sections mounted on slides and rehydrated through a series of xylene, 100, 95 and 70 % v/v ethanol for five minutes each then into distilled water. After flushing with crystal violet solution for 60 s, samples were rinsed with tap water and then flushed with Lugoli's iodine solution for 60 s. Acetone was used to rinse the slide until the acetone ran clear and then the slide was rinsed with tap water. The slide was then counterstained by quickly dipping and removing the slide in eosin solution followed by immediate washing under running tap water to avoid overstraining that affects visualising the Gram stained bacterium. The stained slides were then left to air-dry, and then submerged in xylene for 5 min before a cover slip was applied with DPX mountant. Light microscopy images were captured using a Nikon digital camera and ACT-1 imaging software (Nikon UK Ltd, Surrey, UK).

5.2.13 Enzyme Linked-Immunosorbent Assay (ELISA) quantification of cytokines in supernatant culture media

To assess the immunogenic effects of rhamnolipid and bacteria on pulp tissue, a number of pro-inflammatory, anti-inflammatory and growth factor cytokines were analysed in spent culture supernatant by ELISA. Tooth sections were cultured with bacterial isolates and rhamnolipid as described in section 5.2.12.1 for 24 h. At the end of the incubation period, supernatants were collected, frozen down at -20 °C and allowed to thaw at room temperature when used as below:

5.2.13.1 ELISA quantification of pro-inflammatory cytokines

5.2.13.1.1 TNF-alpha

Secreted TNF-alpha in the collected supernatant was quantified using a sandwich rat Tumour necrosis factor alpha ELISA kit (amsbio, UK). The 96-well plate provided with the kit was pre-coated with rat TNF-alpha antibody. Following the manufacturer's instructions, 50 µL of the cytokine standard were loaded into nominated wells in duplicate in a descending way (640 –

40 ng/L). Forty µL of the sample supernatant was added to the sample wells followed by the addition of 10 µL anti-TNF-alpha biotinylated antibody to the same sample wells. Then, 50 µL streptavidin-HRP were added to the sample wells and standard wells but not to the blank well. The plate was then mixed on a plate mixer, covered with a sealer and incubated for 60 min at 37 °C. At the end of the incubation period, the sealer was removed and the plate was washed 5 times with the wash buffer by soaking the wells with 350 µL wash buffer for 30 s for each wash and blotting the plate onto paper towels after each washing. The plate was then loaded with 50 µL of substrate solution A to each well and then with 50 µL substrate solution B to each well (substrate solutions contain tetramethylbenzidine (TMB)). A new sealer was applied and the plate was incubated for 10 min at 37 °C in the dark. Fifty µL stop solution was added to each well with a change of colour from blue into yellow was evident immediately. Soon (not more than 10 min) after adding the stop solution, the plate optical density was determined at 450 nm using a microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). The amount of TNF-alpha was quantified by plotting the standard samples as a reference standard curve. The experiment was performed in triplicate and the results was expressed in ng/L.

5.2.13.1.2 IL-1beta

The rat IL-1beta sandwich ELISA kit (eBioscience™ Rat IL-1 beta Platinum ELISA Kit) was used to detect and quantify IL-1beta in culture supernatant. The 96-well plate provided with the kit was pre-coated with rat IL-1beta polyclonal antibody. Each sample, blank and standard was assayed in duplicate. According to the manufacturer's instructions, the plate was washed twice with approximately 400 µL wash buffer per well for 10 s each time with thorough aspiration of the microwell contents between washes. Excess wash buffer was removed from the wells by tapping the plate onto an absorbent paper towel. One hundred µL of each standard dilution was added to the corresponding wells in descending manner (2000-31.3 pg/mL). Fifty µL sample diluent was added to the sample wells followed by the addition of another 50 µL of each sample to the sample wells. Biotin-conjugate anti-rat IL-1beta antibody was loaded at 50 µL into each well. The plate was covered with adhesive film and incubated at room temperature for 2 h. The adhesive film was then removed, the wells were emptied and washed 3 times as before. After that, 100 µL of the diluted streptavidin-HRP to all wells including the blank wells. The plate was then covered with a new adhesive film and incubated

for 1 h at room temperature. At the end of the incubation, the film was removed, the wells contents were discarded and wells were washed 3 times as before. One hundred µL of TMB substrate solution were added to all wells and the plate was incubated for 10 min at room temperature protected from direct light exposure. To stop the enzyme reaction, 100 µL stop solution was added just when the highest standard developed a dark blue colour. Absorbance was read at 450 nm immediately after the addition of the stop solution. Standard curve was generated by plotting the absorbance vs. the concentration and a best fit equation was obtained. The concentration read from the curve was multiplied by the dilution factor (x 2) and expressed as pg/mL.

5.2.13.1.3 IL-6

The quantity of the produced IL-6 by the tooth sections into their collected supernatant was quantified using rat IL-6 sandwich ELISA kit (amsbio, UK). The plate provided within the kit was pre-coated with rat IL-6 antibody with 96 wells. Following the manufacturer's instructions, 50 µL of the standard were loaded into the standard designated wells in duplicate in a descending way (24-1.5 ng/L). From this step on to the end of the experiment, the same procedure was followed as in section 5.2.13.1.1.

5.2.13.1.4 IL-18

Rat IL-18 sandwich ELISA kit (amsbio, UK) was used to detect and quantify IL-18 cytokine released into the collected supernatants. A pre-coated 96-well plate with rat IL-18 antibody provided with the kit was employed. The standard dilutions were prepared according to the manufacturer's instructions and 50 µL of each dilution were pipetted into the designated standards wells (400-25 pg/mL). The same steps described in section 5.2.13.1.1 were followed from this step on.

5.2.13.2 ELISA quantification of anti-inflammatory cytokine, transforming growth factor (TGF-beta) and antimicrobial peptide defensin-beta2

5.2.13.2.1 IL-10

IL-10 levels in the collected samples supernatants were determined using a sandwich IL-10 ELISA kit (amsbio, UK). According to the manufacturer's protocol, the rat IL-10 antibody pre-coated plate standards wells were loaded with 50 µL standard dilutions (480-30 pg/mL) in

duplicate. After the addition of the standard dilutions, the same steps were followed as described previously in section 5.2.13.1.1.

5.2.13.2.2 TGF-beta

For accurate detection and quantification of the growth factor TGF-beta in culture supernatants, a rat TGF-beta sandwich ELISA kit (amsbio, UK) was used in this study. The kit was provided with a 96-well microplate pre-coated with rat TGF-beta antibody. The designated standard wells were loaded with 50 µL of the prepared standards dilutions (1200-75 ng/L). The experiment was completed as described in section 5.2.13.1.1.

5.2.13.2.3 Defensin-beta2

The antimicrobial peptide defensin-beta2 was detected in culture supernatants by means of the competitive rat defensin-beta2 ELISA kit (amsbio, UK). Fifty µL of standard dilutions (1200-75 ng/L) and samples were added to the corresponding monoclonal antibody pre-coated wells. Fifty µL biotinylated antigen was added to each well except the blank ones. The plate was covered with a sealer and incubated at 37 °C for 30 min. The wells were then washed off with 300 µL wash buffer five times with decanting the contents and tapping the plate on absorbent paper towel each washing time. Fifty µL of concentrated avidin-HRP were added to the standard and sample wells and the plate was covered and incubated for 30 min at 37 °C. The sealer was removed and the wells were washed as described earlier. Fifty µL of substrate solution A were added to each well and then 50 µL of substrate solution B were added to each well (substrate solutions contain tetramethylbenzidine (TMB)). The plate was covered with a new sealer and incubated for 10 min at 37 c in the dark. Stop solution was added at 50 µL volume to each well and a colour change from blue into yellow was monitored. Immediately after that, the optical density was recorded using a plate reader at 450 nm. Standard curve was generated by plotting the mean absorbance for each standard on the Y-axis against the target antigen concentration on the X-axis. A best fit curve was drawn and the sample defensin-beta2 concentration was obtained.

5.2.14 Effect of serum on rhamnolipid activity on pulp cells

Following culturing rhamnolipid with 20 % serum containg DMEM medium, no rhamnolipid antimicrobial effect was observed indicating a possible serum-binding inactivation. To determine the concentration of foetal bovine serum that can be used in culture medium without binding to and inactivating rhamnolipid, trypan blue viability assay at different FBS %

was performed. Rat dental pulp cells were extracted and cultured as described in section 5.2.1 and 5.2.2. From a frozen vial, cells were cultured at 5000 cell/cm^2 density. From confluent culture flask, cells were harvested, counted and two sets of cells were seeded at 4000 cell/cm^2 density in 12-well plate (SARSTEDT, Germany) using αMEM + 1 % antibiotic + 20, 10, 5, 2 or 0 % FBS for 24 h as shown in table 5.2. At the end of the first incubation period, medium was aspirated. The first set of cells were cultured in αMEM + 1 % antibiotic supplemented with the same concentration of FBS as in the first incubation period and treated with rhamnolipid 0.097 mg/mL (test sample) or without rhamnolipid (control sample) for another 24 h. To account for the residual effect of serum, the second set of cells were cultured in serum-free αMEM + 1 % antibiotic and treated with rhamnolipid 0.097 mg/mL (test sample) or without rhamnolipid (control sample) for another 24 h. Then, the medium was removed, cells were washed with PBS three times and incubated with 200 µL 0.4 % trypan blue stain for 1 min. The stain was removed and the cells were washed once with PBS. The numbers of blue stained and non-stained cells were immediately counted under light microscope. Viability greater than 90 % deemed to be acceptable.

Table 5.2 **Representative table of the two set of cells**

	First 24 h	Second 24 h	
	Cells cultured with αMEM + 1 % antibiotic and FBS	**Control sample** Cells cultured in αMEM + 1 % antibiotic and FBS	**Rhamnolipid treatment** Cells cultured in αMEM + 1 % antibiotic and FBS
First set of cells	20 %	20 %	20 %
	10 %	10 %	10 %
	5 %	5 %	5 %
	2 %	2 %	2 %
	0 %	0 %	0 %
Second set of cells	20 %	0 %	0 %
	10 %	0 %	0 %
	5 %	0 %	0 %
	2 %	0 %	0 %

5.2.15 Statistical analysis

All experiments were subjected to three repeats on separate occasions. Comparison among the different treatment groups was made using Tukey's one way ANOVA multiple comparisons test with post-hoc correction (GraphPad InStat 3 (v3.06)). For DAPI-stained nuclei count, comparison was made to the untreated control of each time point using Dunnett's multiple comparisons one way ANOVA test (GraphPad InStat 3 (v3.06)). A p value of ≤ 0.05 was considered significant (*, $p \leq 0.05$), very significant (**, $p \leq 0.01$) or highly significant (***, $p \leq 0.001$).

5.3 Results

5.3.1 Section a: *Lp*-BS

5.3.1.1 *Lp*-BS cytotoxicity to rat dental pulp cells

Trypan blue viability assay was carried out on extracted rat dental pulp cells following 24 h exposure to *Lp*-BS at three concentrations (20, 10 and 5 mg/mL). At 20 mg/mL, only few cells remained attached to the well surface after 24 h and they all picked up the dye and stained blue indicating their mortality in response to the treatment when compared to the control group (Figure 5.2). Cells treated with the lower concentrations of 10 and 5 mg/mL did not retain the stain fully resulting in difficulty of distinguishing the dead from the living cells when examined at 10x magnification (Figure 5.2). At higher magnification (20x), some of these cells demonstrated intra-cellular bodies or structures (Figure 5.2, red arrows) which might be a cytoplasmic vacuolation or chromatin condensation characteristics of apoptotic cells (Korsnes 2012).

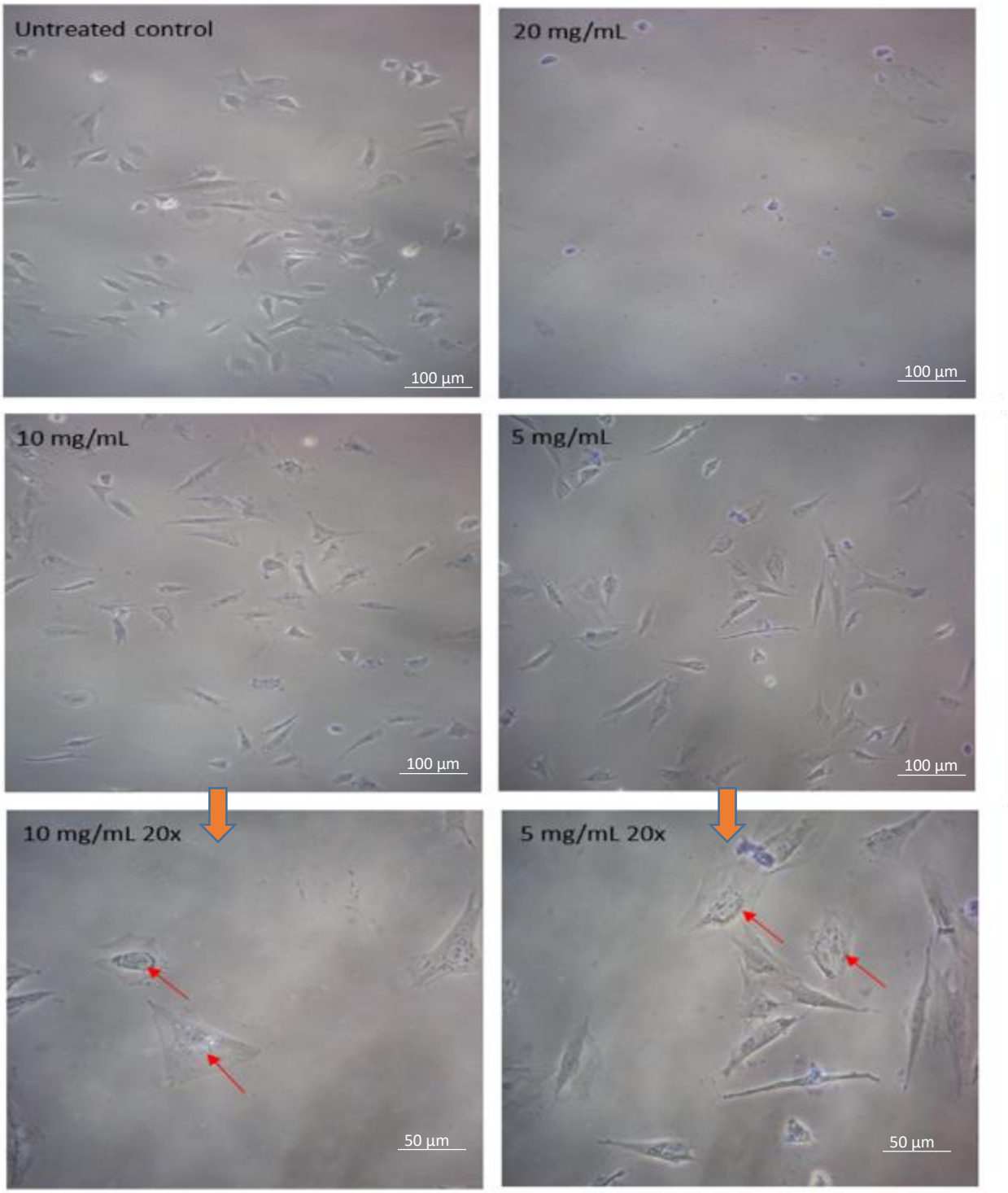


Figure 5.2 ***Lp*-BS *in-vitro* cytotoxic effect on rat dental pulp cells.** Trypan blue staining was employed to determine viability at 24 h post-treatment. 20 mg/mL *Lp*-BS was found toxic (no viable cells were noticed). At 10 mg/mL and 5 mg/mL, cells did not stain properly by trypan blue and it was difficult to distinguish life and dead cells based on colour using power 10x magnification. At higher magnification 20x, cells showed intra-cellular structures or bodies (red arrows) which might be apoptotic bodies. The images are representative of three independent experiments.

5.3.1.2 Apoptotic effect of *Lp*-BS on rat dental pulp cells

To account for the apoptotic potential of *Lp*-BS, a TUNEL assay was performed on rat dental pulp cells grown with 10 and 5 mg/mL *Lp*-BS for 24, 48 and 72 h (Figure 5.3). Subsequent to incubation with 10 mg/mL *Lp*-BS for 24 h, a high percentage (> 30 %) of the pulp cells was found to be TUNEL-positive. This was significantly ($p < 0.001$) higher than the counterpart untreated control (Figure 5.4). The total number of DAPI-stained nuclei was significantly ($p < 0.001$) lower than the untreated control of the 24 h time point (Figure 5.5). Treatment with 5 mg/mL of *Lp*-BS for 24 h did not show significant ($p > 0.05$) high percentage of apoptotic cells when compared to the untreated control (Figure 5.4) and the number of DAPI-nuclei was found comparable to the untreated control ($p > 0.05$) (Figure 5.5). Further incubation of cells with 10 and 5 mg/mL for 48 and 72 h did not induce significant ($p > 0.05$) percentage of apoptotic TUNEL-positive cells compared to the untreated controls (Figure 5.4). However, total cell number of DAPI-stained nuclei treated with 5 and 10 mg/mL for 48 and 72 h decreased dramatically ($p < 0.001$) over time to approach close to zero after 72 h (Figure 5.5). See appendix II for magnified photomicrographs.

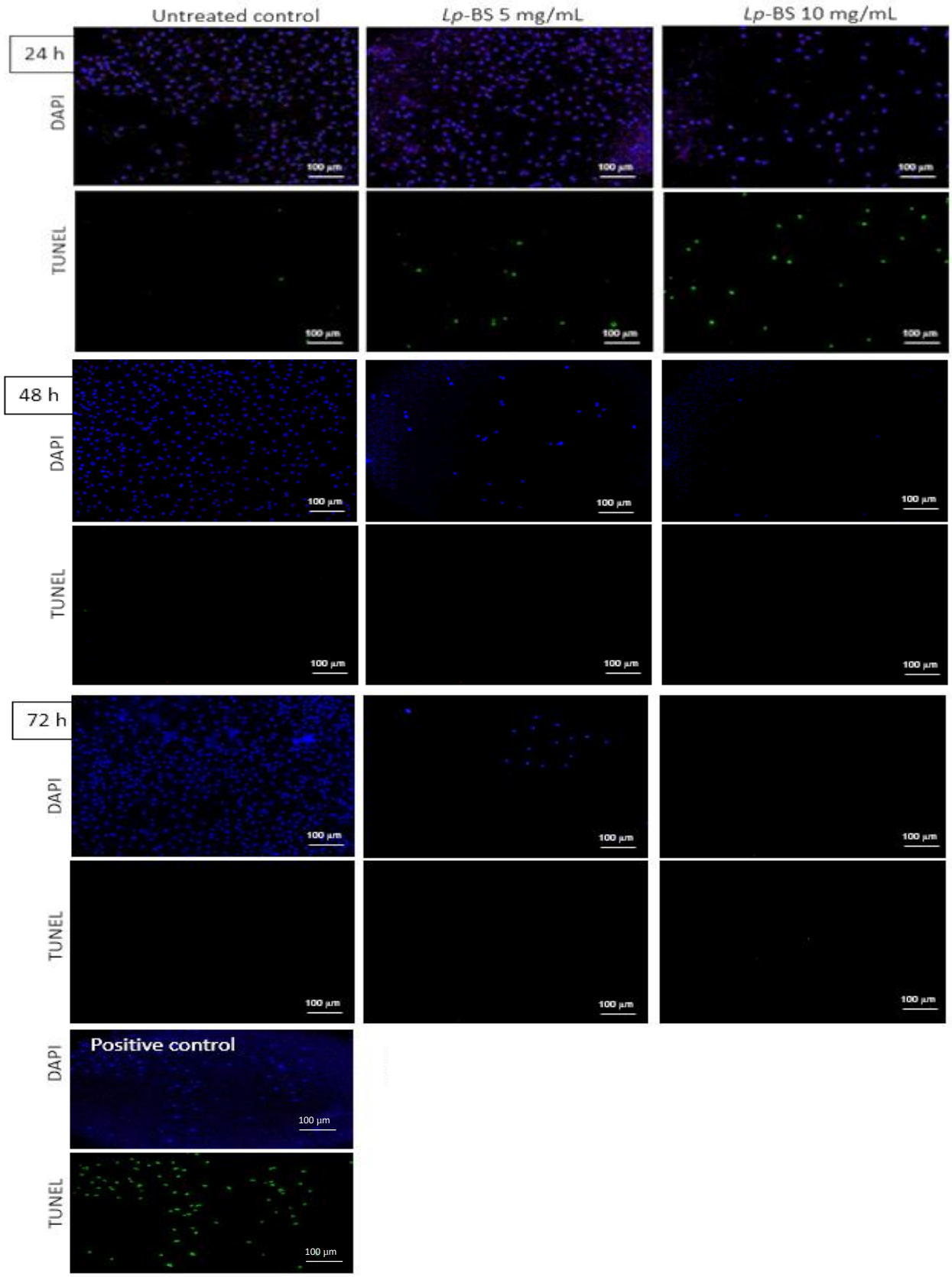


Figure 5.3 **Apoptotic effect of *Lp*-BS**. To determine the apoptotic potential of *Lp*-BS, TUNEL assay was performed on pulp cells grown *in-vitro* following treatment with 10 and 5 mg/mL for 24, 48 and 72 h. The images are representative of 3 independent experiments. See appendix II for magnified photomicrographs.

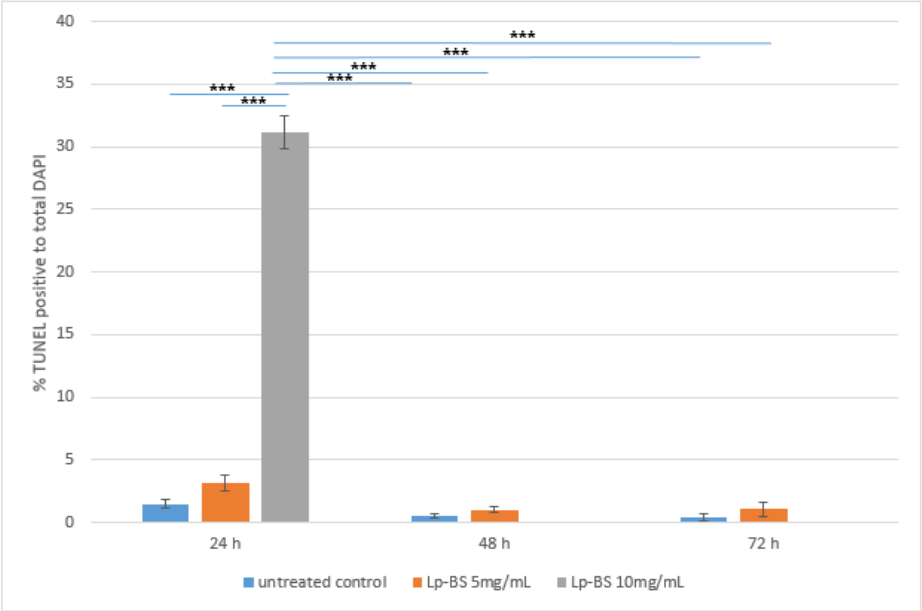


Figure 5.4 **Percentage of apoptotic cells to the total cells number following treatment with *Lp*-BS 5 and 10 mg/mL**. Comparison was made using Tukey's one way ANOVA multiple comparisons test. ***; $p < 0.001$. Error bar represent SEM of three independent experiments. N=3.

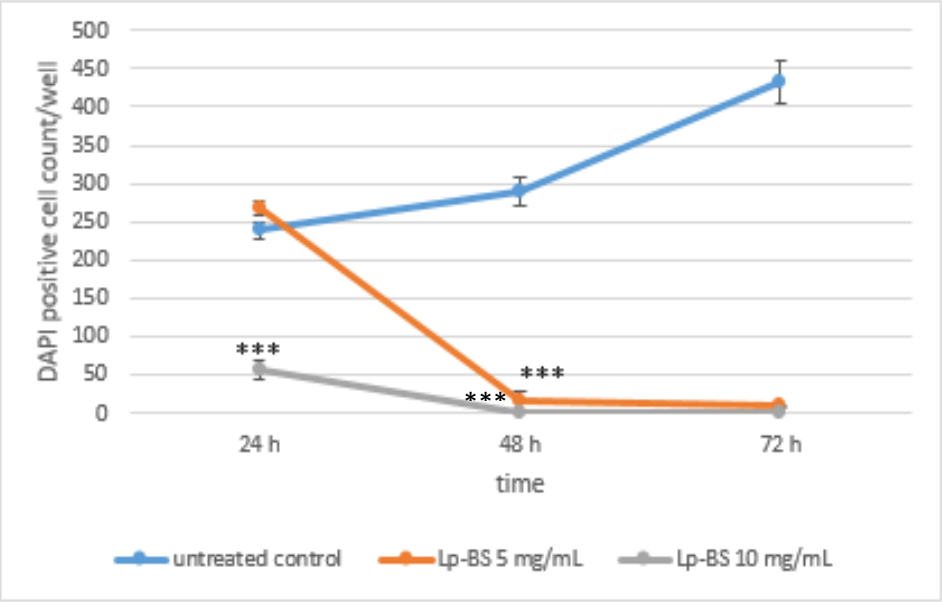


Figure 5.5 **Rat dental pulp cells count following treatment with *Lp*-BS for 24, 48 and 72 h.** The DAPI-stained nuclei were counted. Comparison was made to the untreated control of each time point using Dunnett's multiple comparisons one way ANOVA test. ***; $p < 0.001$. Error bar represent SEM of three independent experiments. N=3.

5.3.1.3 Viability of rat pulp tissue following treatment with *Lp*-BS solutions

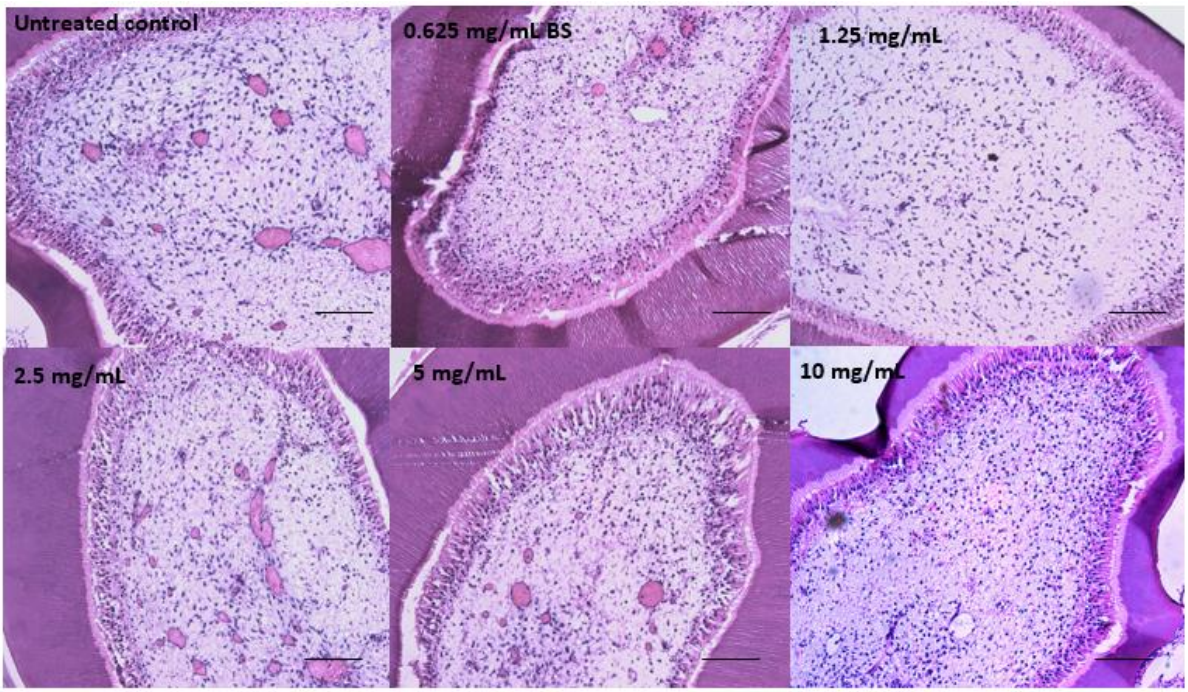
Tooth slices were cultured with a series of *Lp*-BS concentrations (10, 5, 2.5, 1.25 and 0.625 mg/mL) for 24 h and with 0.625 and 10 mg/mL *Lp*-BS for 48 h. These sections were processed automatically and stained with H&E stain. As was seen under standard culture conditions, tissue architecture and morphology was maintained for the first 24 h culture with the *Lp*-BS five concentrations (Figure 5.6). Areas of both dentine and pre-dentine could be observed with odontoblasts remaining as tall columnar cells, the viability of which can be seen from the darkly stained purple nuclei. Healthy pulp fibroblasts were seen as spindle shaped cells with dark purple nuclei. Staining of the matrix was seen between the cells, all of which were indicators that the tooth slices were surviving in the biosurfactant solutions with no deleterious effects (Figure 5.6a). This was confirmed using cell counts on the pulp layer which were performed using ImageJ software. One-way Tukey's ANOVA test showed no significant decrease ($p > 0.05$) in cell number at 24 h incubation with any of the tested *Lp*-BS solutions compared to the untreated control (Figure 5.6b).

Stained sections which were treated with 0.0625 mg/mL *Lp*-BS for 48 h demonstrated no change to the untreated control sections in tissue architecture and morphology of the dentine, pre-dentine, odontoblasts and pulp fibroblast cells (Figure 5.7). Cell counts on the pulp area also revealed no significant difference ($p > 0.05$) to the untreated 48 h-old control section (Figure 5.9).

Incubation of the tooth sections with 10 mg/mL for 48 h appeared to be associated with a breakdown of the surrounding extracellular matrix (Figure 5.8 red arrows). This matrix degradation was seen in the photomicrographs as a decrease in the structural integrity of the tissues as there was less staining seen surrounding the cells where there had previously been staining. The odontoblasts layer seemed to be affected by the toxic effect of biosurfactant as demonstrated in the photomicrographs as loss and fading of the nuclei—karyolysis of the majority of the odontoblast cells following 48 h culture with 10 mg/mL *Lp*-BS (Figure 5.8 black arrows). Cellular toxicity was also obvious in the pulp region. The majority of the pulp tissue cells underwent karyolysis evident in the photomicrographs by loss and fading of nuclei (Figure 5.8 blue arrows). Apoptotic cell death was also noticed as fragmented basophilic nuclei-karyorrhexis in the pulp tissue region (Figure 5.8 green circle) (Elmore 2007). The observed changes in cell number which were seen in the histological sections was confirmed

using average cell counts over a 50 µm² area. These counts demonstrated that there was a significant ($p < 0.001$) decrease in pulpal cell number after 48 h culture with 10 mg/mL when compared to the untreated control sections (Figure 5.9).

a-



b-

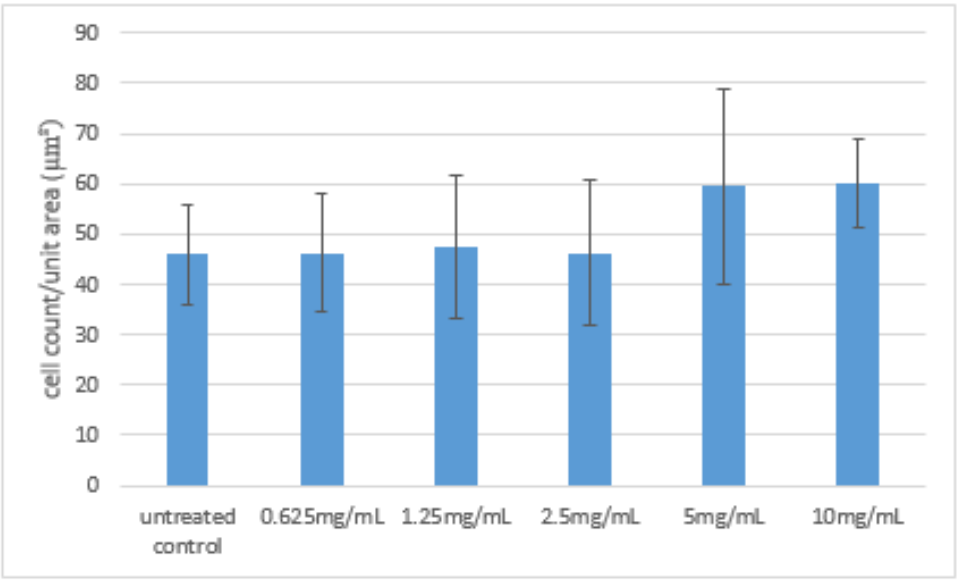


Figure 5.6 **Viability of rat pulp tissue follow treatment with *Lp*-BS.** Although *Lp*-BS was shown to be toxic against cells, the viability of pulp tissue was assayed in the presence of different concentrations (10, 5, 2.5, 1.25 and 0.625 mg/mL) of *Lp*-BS for 24 h. **a-** Tooth slices fixed, processed and histological sections stained with H&E stain. Nuclei of the pulp area excluding the odontoblast layer were counted automatically using ImageJ software divided by the surface area. **b-** Using Tukey's one-way ANOVA, no significant difference was obtained among the different concentrations and the untreated control ($p > 0.05$). Images were obtained at 20x magnification. Error bars represent SEM of three independent experiments. Scale bar=100 µm.

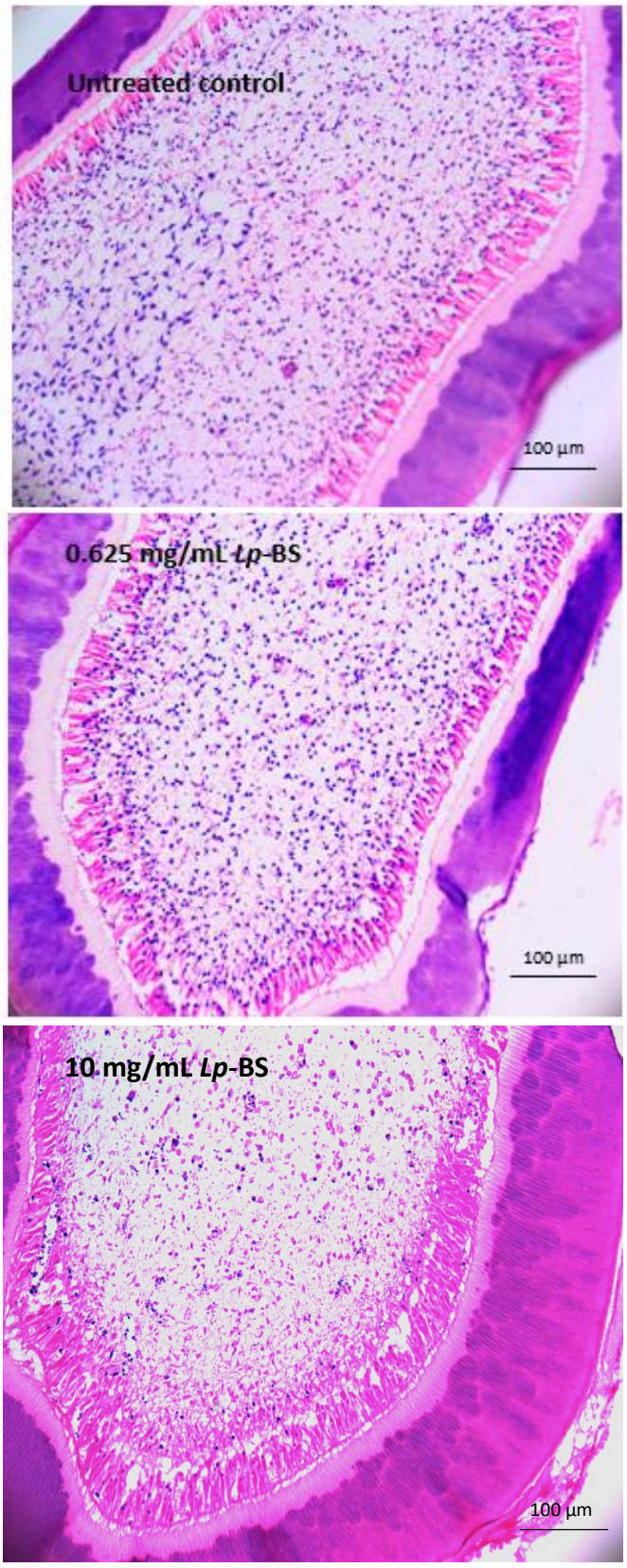


Figure 5.7 **Viability of rat pulp tissue follow treatment with *Lp*-BS for 48 h.** Although *Lp*-BS was shown to be toxic against cells, the viability of pulp tissue was assayed in the presence of 10 and 0.625 mg/mL of *Lp*-BS for 48 h. Tooth slices were fixed and processed and histological sections stained with H&E stain. Images obtained at 20x magnification. N=3.

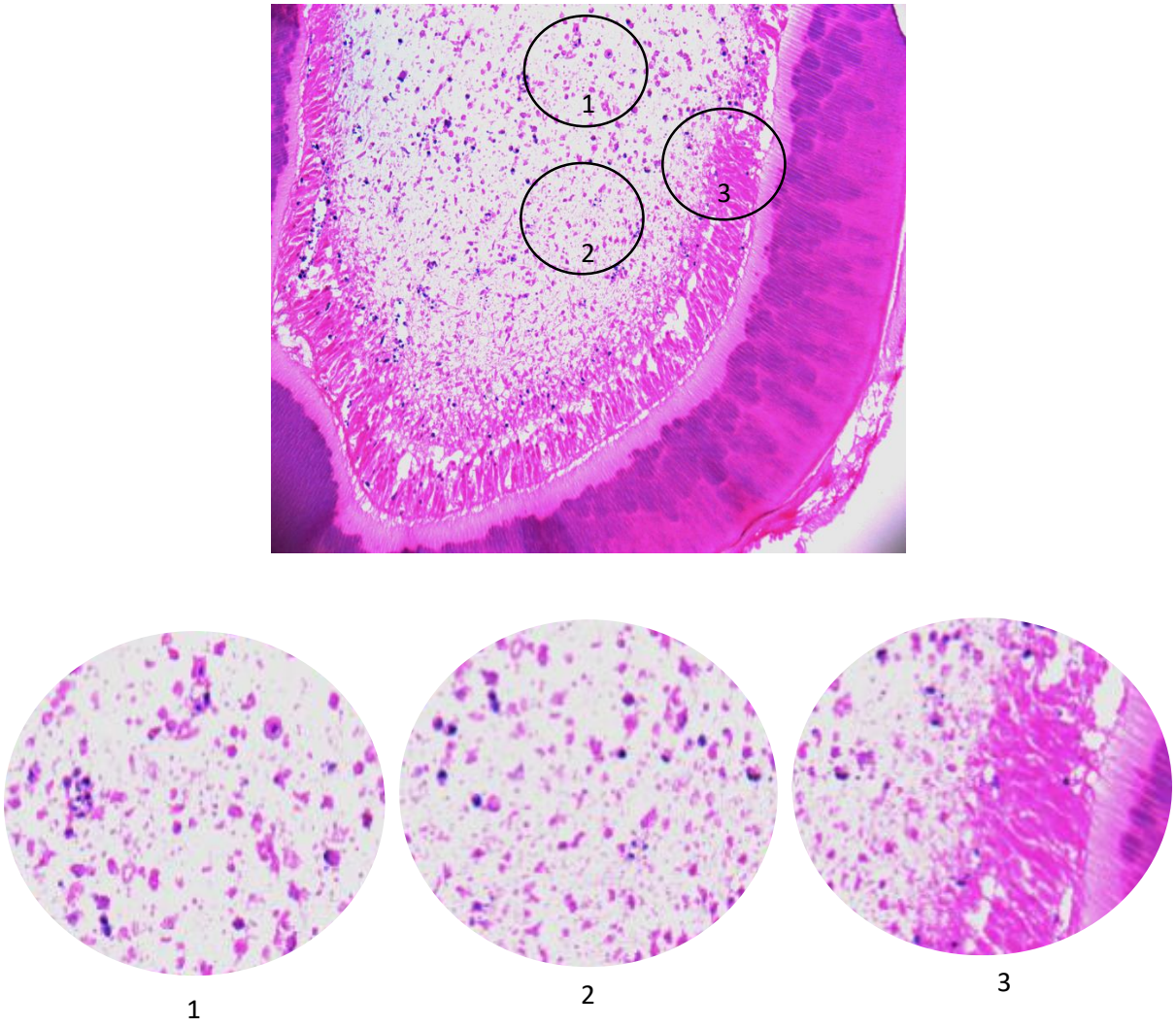


Figure 5.8 **Photomicrograph representation of tooth section treated with *Lp*-BS 10 mg/mL for 48 h.** Circle #1 represents the breakdown of the surrounding extracellular matrix and the loss and fading of the nuclei—karyolysis of the majority of the pulp tissue cells. Circle #2 represents fragmented basophilic nuclei-karyorrhexis characteristic of apoptosis. Circle #3 represents the loss and fading of the nuclei—karyolysis of the majority of the odontoblast cells.

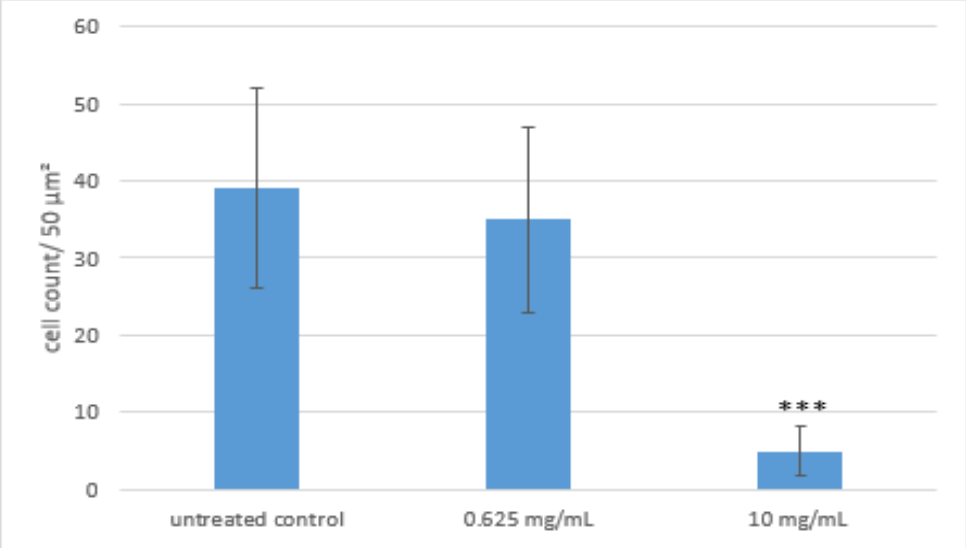


Figure 5.9 **Cells count of the pulp tissue following treatment with *Lp*-BS for 48 h.** Statistical analysis was performed using Tukey's one-way ANOVA comparisons test. ***; $p < 0.001$. Error bar represent SEM of three independent experiments.

5.3.1.4 RT-qPCR of cytokines

5.3.1.4.1 Concentration of mRNA

Lp-BS treated pulp tissue was extirpated and mRNA was extracted. mRNA concentration and purity were determined using a NanoVue 463 Spectrophotometer (GE Healthcare Life Sciences, Buckinghamshire, UK). mRNA concentrations extracted from tooth sections treated with 10 mg/mL *Lp*-BS showed a trend of significant decline with time from > 100 ng/µL after 2 and 6 h incubation to about 40 ng/µL after 24 h incubation (Figure 5.10). Statistical analysis using Tukey's one-way ANOVA revealed that the mRNA yield obtained from the untreated control tooth sections and those treated with 0.625 mg/mL *Lp*-BS at the three time points was significantly ($p < 0.05$) higher than that of the samples treated with the high concentration of 10 mg/mL *Lp*-BS (Figure 5.10).

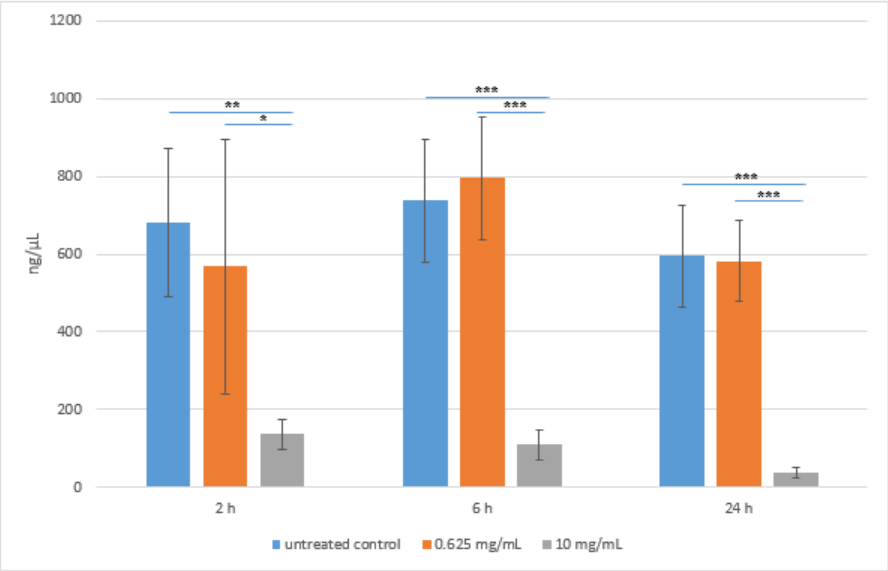


Figure 5.10 **mRNA concentration of samples treated with *Lp*-BS.** Tukey's one-way ANOVA revealed a significant reduction of the obtained mRNA level over time when the pulp tissue was treated with 10 mg/mL *Lp*-BS. Error bar represent SEM of three independent experiments.

5.3.1.4.2 Inflammatory markers

For further characterisation of *Lp*-BS inflammatory potential on pulp tissue cells, mRNA gene expression of a series of inflammatory cytokines was assayed over 2, 6 and 24 h incubation with 0.625 or 10 mg/mL *Lp*-BS.

5.3.1.4.2.1 Gene expression profile of pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-6)

5.3.1.4.2.1.1 TNF-alpha

Tooth sections co-incubated with 10 mg/mL for 2 h demonstrated a significant ($p < 0.05$) down regulation of TNF-alpha mRNA expression when compared to the untreated control. Further incubation for 6 and 24 h also resulted in down regulated expression of TNF-alpha though not significantly in comparison to the control ($p > 0.05$) (Figure 5.11). Co-culture with the low concentration of *Lp*-BS (0.625 mg/mL) brought TNF-alpha gene expression below the control level 2 and 6 h. Continuing treatment with 0.625 mg/mL for 24 h caused a significant ($p <$

0.05) 1.7 fold increase in the expression of TNF-alpha when compared to the untreated control (Figure 5.11).

5.3.1.4.2.1.2 IL-1beta

IL-1beta gene expression was elevated in response to treatment for 2, 6 and 24 h with the low concentration of 0.625 mg/mL but not at 10 mg/mL *Lp*-BS in comparison to the untreated control ($p < 0.001$). Although the mRNA expression of the pro-inflammatory cytokine IL-1beta underwent significant ($p < 0.001$) up-regulation in response to treatment with 0.625 mg/mL *Lp*-BS, the fold change decreased over time from 8.1 and 6.3 after 2 h and 6 h respectively to reach 3.6 at the end of incubation (24 h) (Figure 5.11). Treatment with 10 mg/mL did not show a significant effect on gene expression of the target cytokine. After 2 h culture of the tooth slice with 10 mg/mL *Lp*-BS, there was a non-significant ($p > 0.05$) down regulation of IL-1beta mRNA expression when compared to the untreated control. The expression up-regulated slightly ($p > 0.05$) in the following 6 h in co-culture with *Lp*-BS (1.4 fold) to be tapered down again at the end of the experiment (24 h), though insignificantly ($p > 0.05$).

5.3.1.4.2.1.3 IL-6

Lp-BS treatment at 0.625 mg/mL of tooth section resulted in up-regulation of mRNA IL-6 expression by 2.6 fold after 2 h incubation, maximised after 6 h to 6.1 fold and then declined to 4.1 fold of the control. It was statistically significant at 6 and 24 h ($p < 0.001$) in comparison to the untreated control (Figure 5.11). Of the samples treated with 10 mg/mL, IL-6 gene expression maximised significantly only after 6 h incubation ($p < 0.001$ in comparison to the control) reaching a comparable level to that induced by 0.625 mg/mL (i.e. 6.8 fold increase). The expression then declined significantly ($p < 0.001$ in comparison to 6 h time point) after 24 h while no expression was reported at 2 h incubation when compared to the untreated control (Figure 5.11).

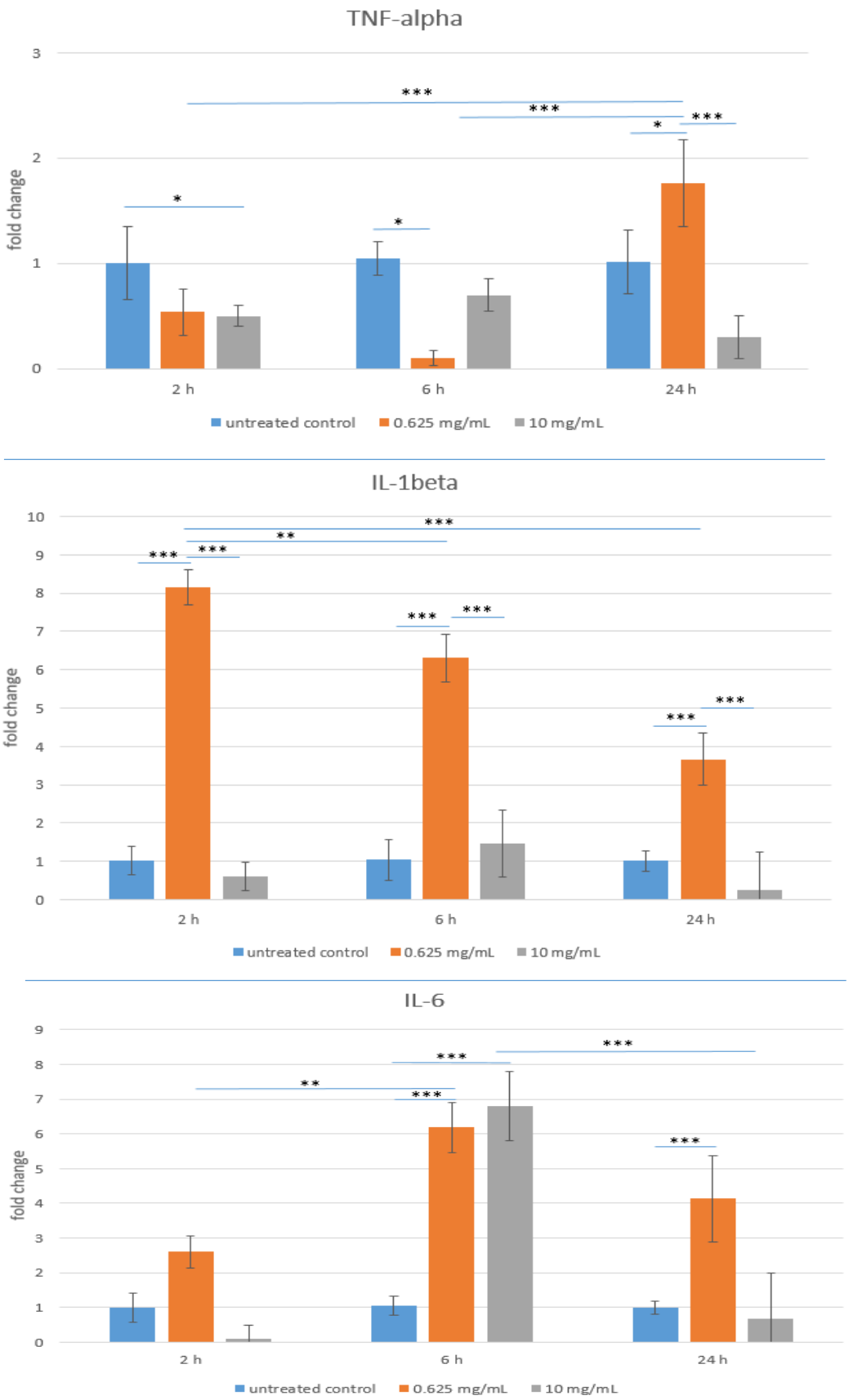


Figure 5.11 **Pro-inflammatory genes expression in response to *Lp*-BS treatment.** Tukey's one way ANOVA was used to determine significant difference among the different groups. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$. Error bars represent SEM of three independent experiments. Note: B2M was used as reference gene.

5.3.1.4.2.2 Gene expression profile of anti-inflammatory cytokines (IL-4 and IL-10)

5.3.1.4.2.2.1 IL-4

Only 0.625 mg/mL *Lp*-BS demonstrated the potential to significantly ($p < 0.001$) induce IL-4 mRNA up-regulation by 3.5 fold after 24 h incubation with the tooth section when compared to the untreated control. However, at 2 and 6 h, the target gene was knocked down by the effect of *Lp*-BS at the two concentrations tested (0.625 and 10 mg/mL) (Figure 5.12).

5.3.1.4.2.2.2 IL-10

Statistically significant ($p < 0.001$) inductions of IL-10 mRNA expression by 11.6, 18.2 and 10.3 folds to the control were observed in tooth section cultured with 0.625 mg/mL *Lp*-BS at 2, 6 and 24 h, respectively (Figure 5.12). In comparison, tissue treated with 10 mg/mL induced up-regulation of the IL-10 gene by only 2.9 and 1.7 folds after 6 and 24 h incubation respectively, though this induction was not statistically significant ($p > 0.05$) when compared to the untreated controls (Figure 5.12).

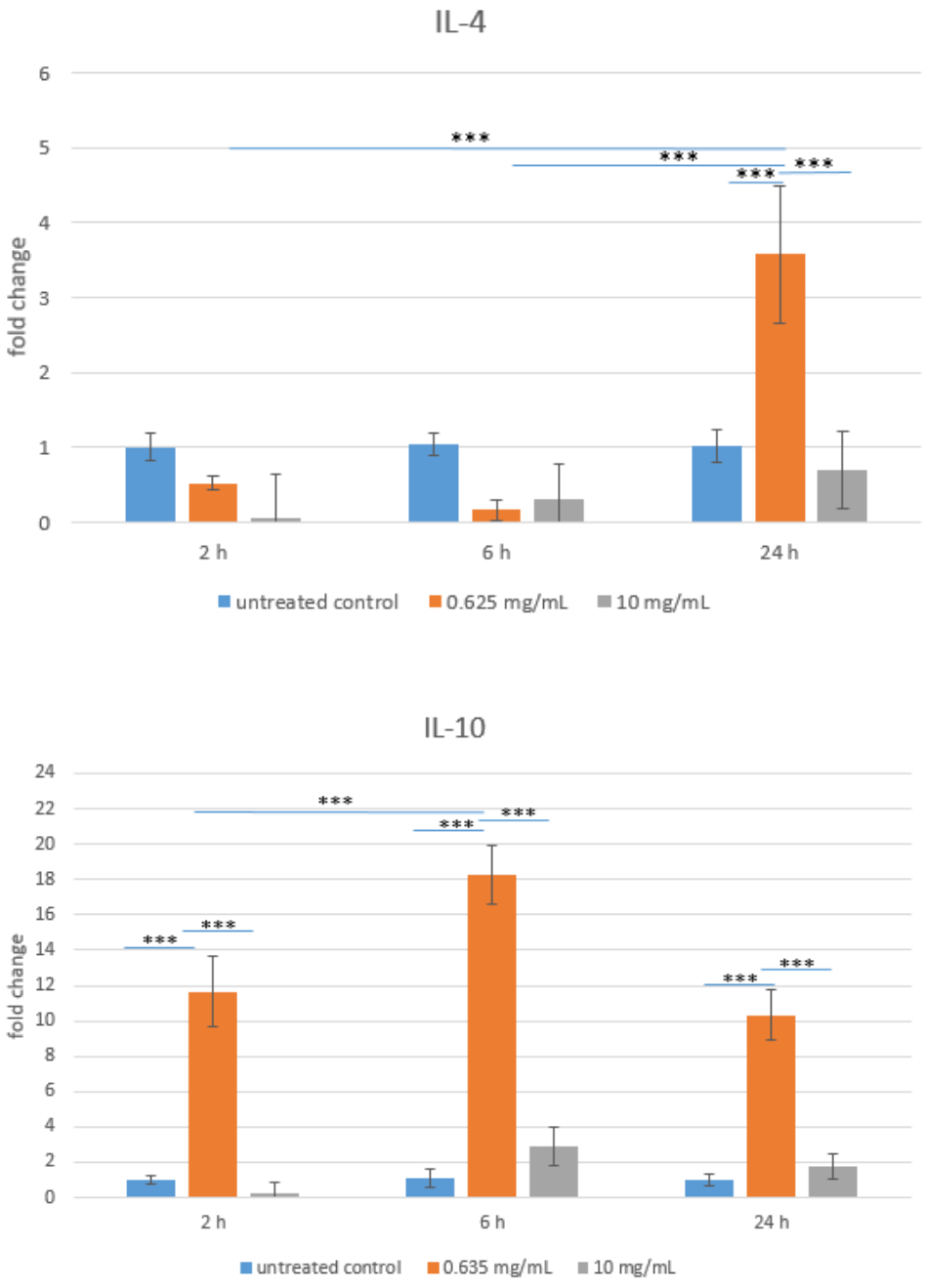


Figure 5.12 **Anti-inflammatory genes expression (IL-4 and IL-10) in response to treatment with *Lp*-BS.** Tukey's one way ANOVA was used to determine significant difference among the different groups. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$. Error bars represent SEM of three independent experiments. Note: B2M was used as reference gene

5.3.1.4.2.3 Gene expression profile of TGF-beta and beta defensin cytokines

5.3.1.4.2.3.1 TGF-beta1

A down regulation of the mRNA expression of TGF-beta1 was evident for the samples treated with 0.625 and 10 mg/mL at 2, 6 and 24 h (Figure 5.13). Co-culture of the tooth slice with 10 mg/mL *Lp*-BS down regulated TGF-beta1 gene expression significantly by 0.2, 0.2 and 0.06 folds at 2, 6 and 24 h incubation respectively ($p < 0.05, 0.01, 0.001$ respectively) as shown in figure 5.12. Co-culture of the tooth slice with 0.625 mg/mL knocked down TGF-beta1 gene expression which was statistically significant ($p < 0.01$) at 6 h incubation (Figure 5.13) but not at 2 or 24 h ($p > 0.05$).

5.3.1.4.2.3.2 Defensin beta2

Treatment with 10 mg/mL caused down regulation of the antimicrobial defensin beta2 mRNA expression at all of the time points (2, 6 and 24 h) though insignificantly ($p > 0.05$) (Figure 5.13). Similarly, co-culture of the tooth slices with 0.625 mg/mL down regulated the target gene expression at 2 and 6 h but the reduction was insignificant ($p > 0.05$). Interestingly, a significant up-regulation by 5.4 fold ($p < 0.001$) of the defensin beta2 mRNA expression was induced by treatment with 0.625 mg/mL after 24 h incubation (Figure 5.13).

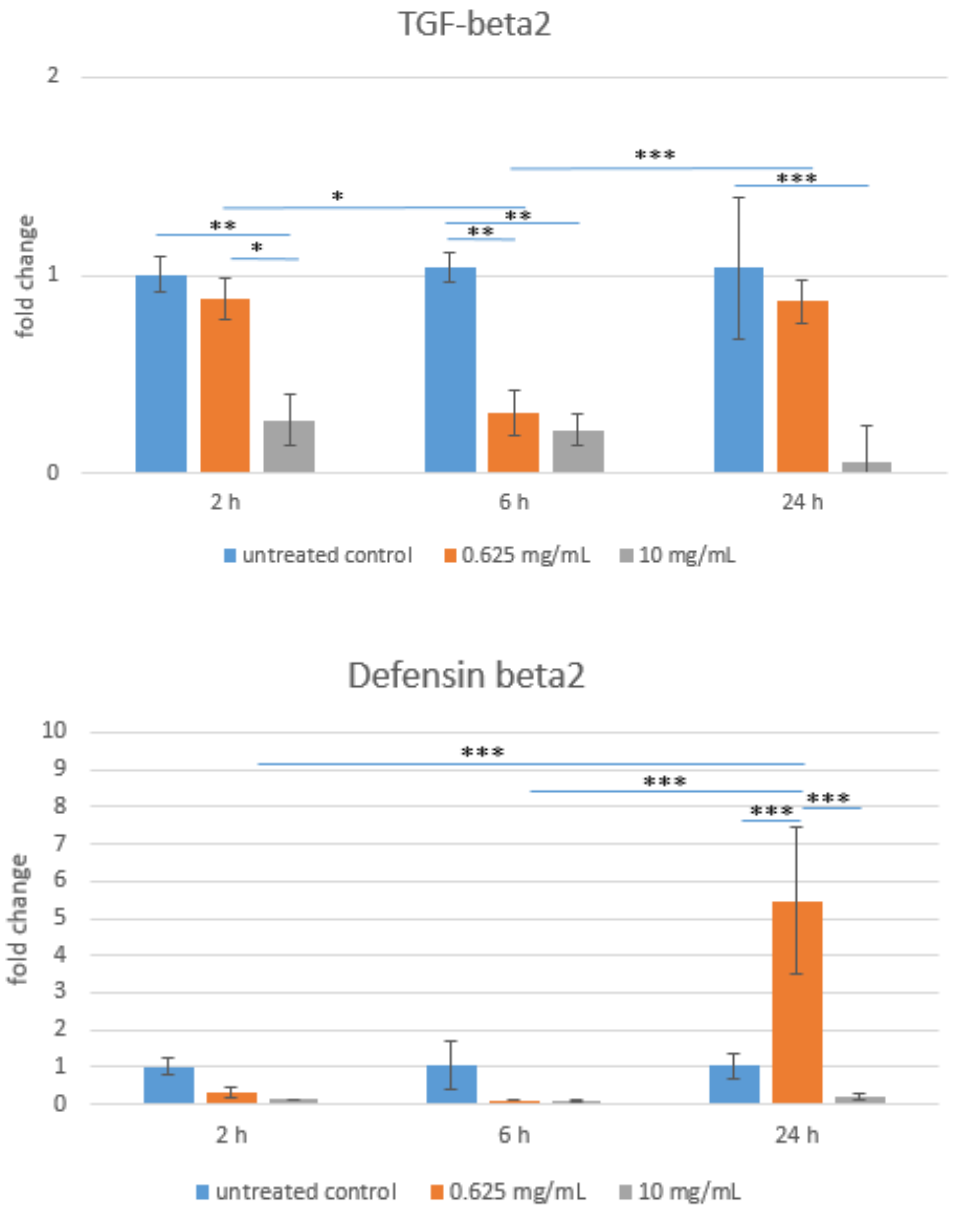


Figure 5.13 **TGF-beta1 and defensin beta2 gene expression in response to treatment with *Lp*-BS.** Tukey's one way ANOVA was used to determine significant difference among the different groups. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$. Error bars represent SEM of three independent experiments. Note: B2M was used as the reference gene.

5.3.1.4.3 Apoptotic marker gene expression in response to treatment with *Lp*-BS

Figure 5.14 highlights *Lp*-BS-induced alterations in the mRNA expression of markers associated with apoptosis after 24 h. The overall picture of *Lp*-BS induced apoptotic effect demonstrated a linearity in pulp tissue response to the low and high *Lp*-BS concentrations tested. Of the pro-apoptotic genes tested (casp3, casp8 and FAS), casp8 and FAS were up-regulated significantly in response to treatment with *Lp*-BS when compared to the untreated control. Casp8 gene expression was up-regulated by 2.5 and 3.1 fold when co-cultured for 24 h with 0.625 and 10 mg/mL respectively ($p < 0.05$ and 0.01 respectively) relative to the untreated control sample. A relative fold change increase of 2.3 and 4.5 was reported for FAS gene in response to treatment of the tooth slices with 0.625 and 10 mg/mL respectively ($p < 0.05$ and 0.001 respectively in comparison to the control). The gene expression of casp3 was not affected by *Lp*-BS 0.625 and 10 mg/mL ($p > 0.05$). *Lp*-BS demonstrated the potential to induce the expression of the anti-apoptotic Bcl2 gene by 1.5 and 3.7 folds at 0.625 and 10 mg/mL *Lp*-BS respectively ($p < 0.05$ and 0.001 respectively) when compared to the untreated control.

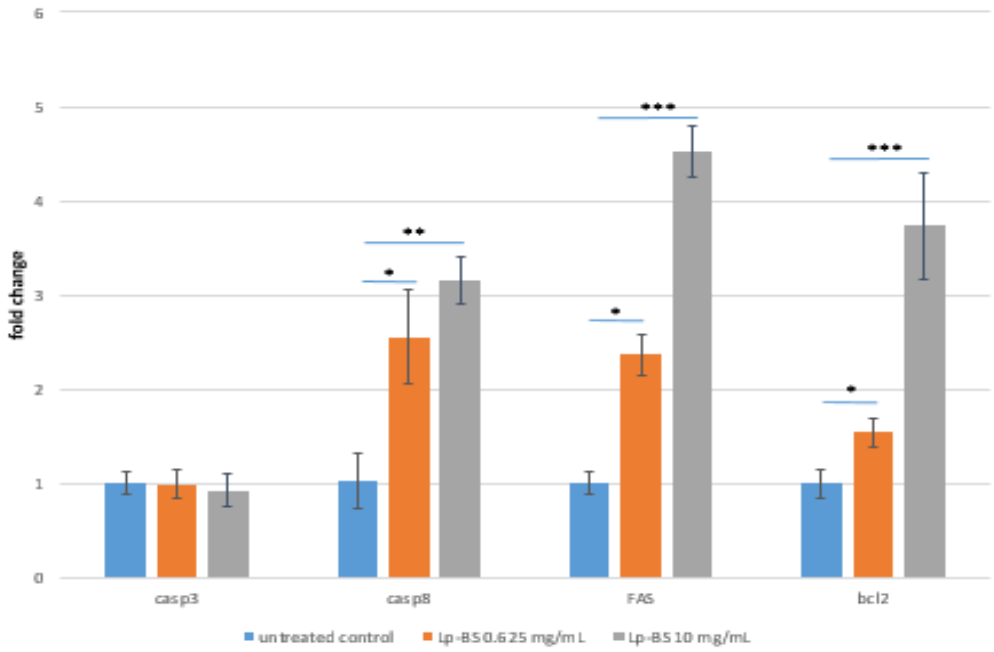


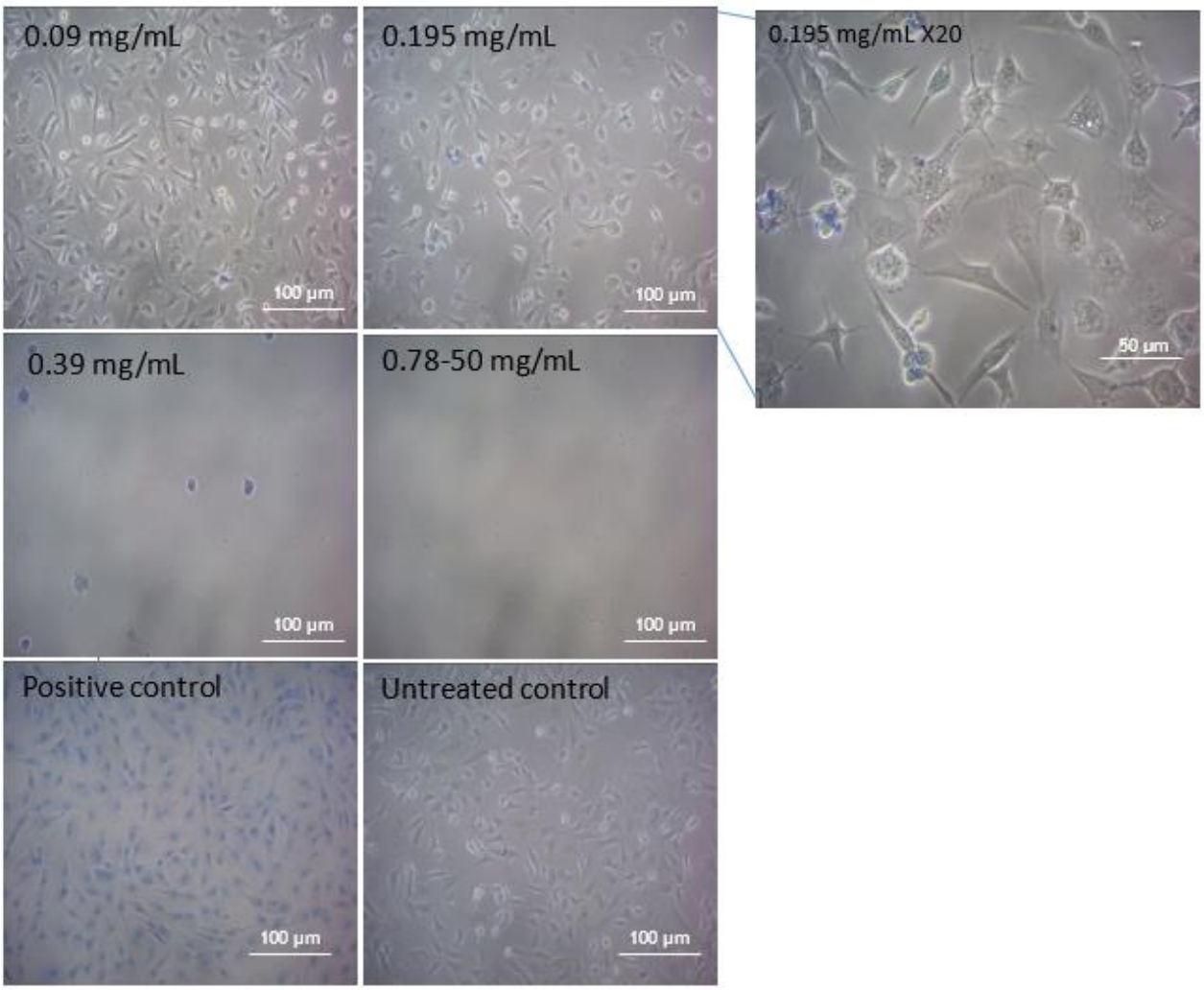
Figure 5.14 **Apoptosis markers mRNA expression in response to treatment with *Lp*-BS for 24 h.** Tukey's one way ANOVA was used to determine significant difference among the different groups. *; $P < 0.05$, **; $p < 0.01$, ***; $p < 0.001$. Error bars represent SEM of three independent experiments. Note: B2M was used as the reference gene.

5.3.2 Section b: rhamnolipid

5.3.2.1 Rhamnolipid cytotoxicity on rat dental pulp cells in 10% serum-containing medium

To determine the viability of extracted rat dental pulp cells when exposed to rhamnolipid, trypan blue viability assay was performed. Different concentrations of rhamnolipids were tested (50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.625, 0.39, 0.195 and 0.097 mg/mL) as demonstrated in figure 5.15a. Percentages of viable cells to the total cells count are presented in figure 5.15b. Concentrations from 0.39 up to 50 mg/mL showed 100 % toxicity (i.e. no viable cell remained). More than 90 % cell viability was reported when cells exposed to rhamnolipid 0.195 mg/mL. The morphology of the cells treated with 0.195 mg/mL rhamnolipid did not seem normal in comparison to the untreated control cells. At 20x magnification using light microscopy, these cells demonstrated intra-cellular vesicles or bodies which might be a pre-apoptotic bodies (Figure 5.15a). Cells grown at 0.097 mg/mL showed more than 95 % viability and no intracellular bodies were detected (Figure 5.15b).

a-



b-

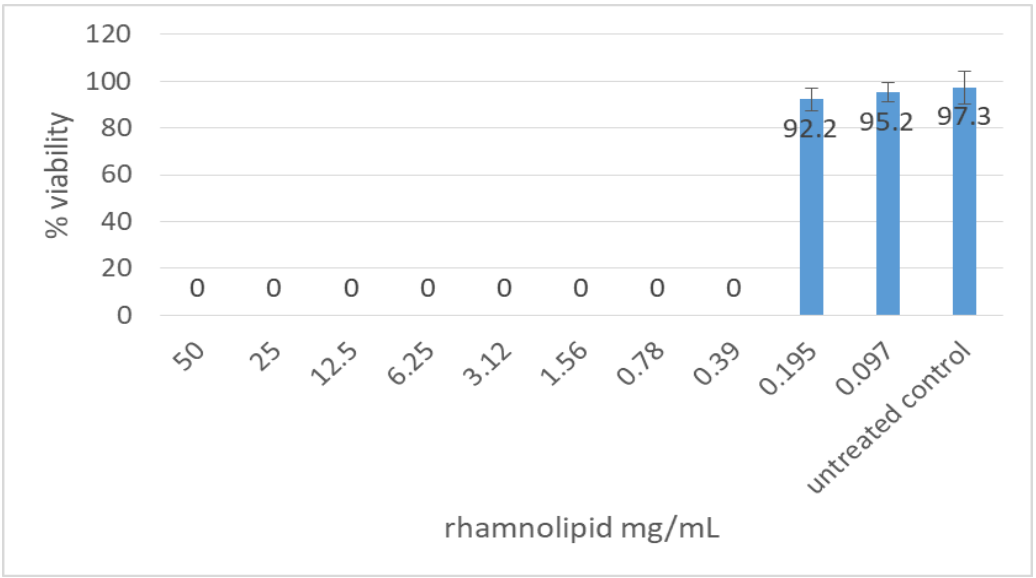


Figure 5.15 **Viability of rat dental pulp cells in response to treatment with rhamnolipid. a-** Photomicrographs are representative of three independent repeats. **b-** Percentage of cell viability. Error bars represent SEM of three independent repeats.

5.3.2.2 Apoptotic effect of rhamnolipid on rat dental pulp cells

To determine if rhamnolipid at 0.097 and 0.195 mg/mL had the ability to induce apoptosis in dental pulp cells, a TUNEL assay was employed. Cells were cultured with either 0 mg/mL (control), 0.097 mg/mL or 0.195 mg/mL rhamnolipid for 24, 48 and 72 h as depicted in the micro-images of figure 5.16.

At the lower rhamnolipid concentration (0.097 mg/mL), a significantly ($p < 0.001$) high percentage (> 16 %) of TUNEL-positive cells were reported after 24 h incubation with the treatment (Figure 5.17) in comparison to the untreated control. More than 6 % of the cells treated with 0.195 mg/mL rhamnolipid for 24 h was found TUNEL-positive compared to the untreated control cell sample ($p < 0.001$)(Figure 5.17). DAPI-positive nuclei count of the 0.195 mg/mL rhamnolipid treated cells was lower than the untreated control but the difference was not significant ($p > 0.05$). Counts of DAPI-positive nuclei of cells treated with 0.097 mg/mL demonstrated no difference to the untreated control ($p > 0.05$) (Figure 5.18). Incubation for 48 h with the treatment (0.097 and 0.195 mg/mL), the percentage of apoptotic cells dropped dramatically showing no significant difference ($p > 0.05$) to the untreated control (Figure 5.17).

The number of DAPI-positive nuclei of 0.097 and 0.195 mg/mL rhamnolipid-treated cells decreased significantly ($p < 0.05$) at 48 h post treatment (Figure 5.18). Further incubation of the cells with rhamnolipid solutions of 0.097 and 0.195 mg/mL for 72 h resulted in less than 1 % apoptosis (Figure 5.17) which was comparable to the untreated control ($p > 0.05$). Cells treated with 0.097 mg/mL showed the potential to regenerate and at the end of the 72 h incubation their DAPI count was close to the untreated control, though the difference was still significant ($p < 0.05$) (Figure 5.18). DAPI-positive cells grown with 0.195 mg/mL for 72 h continued to decrease reaching a significant low level ($p < 0.001$) in comparison to the control sample (Figure 5.18). See appendix II for magnified photomicrographs.

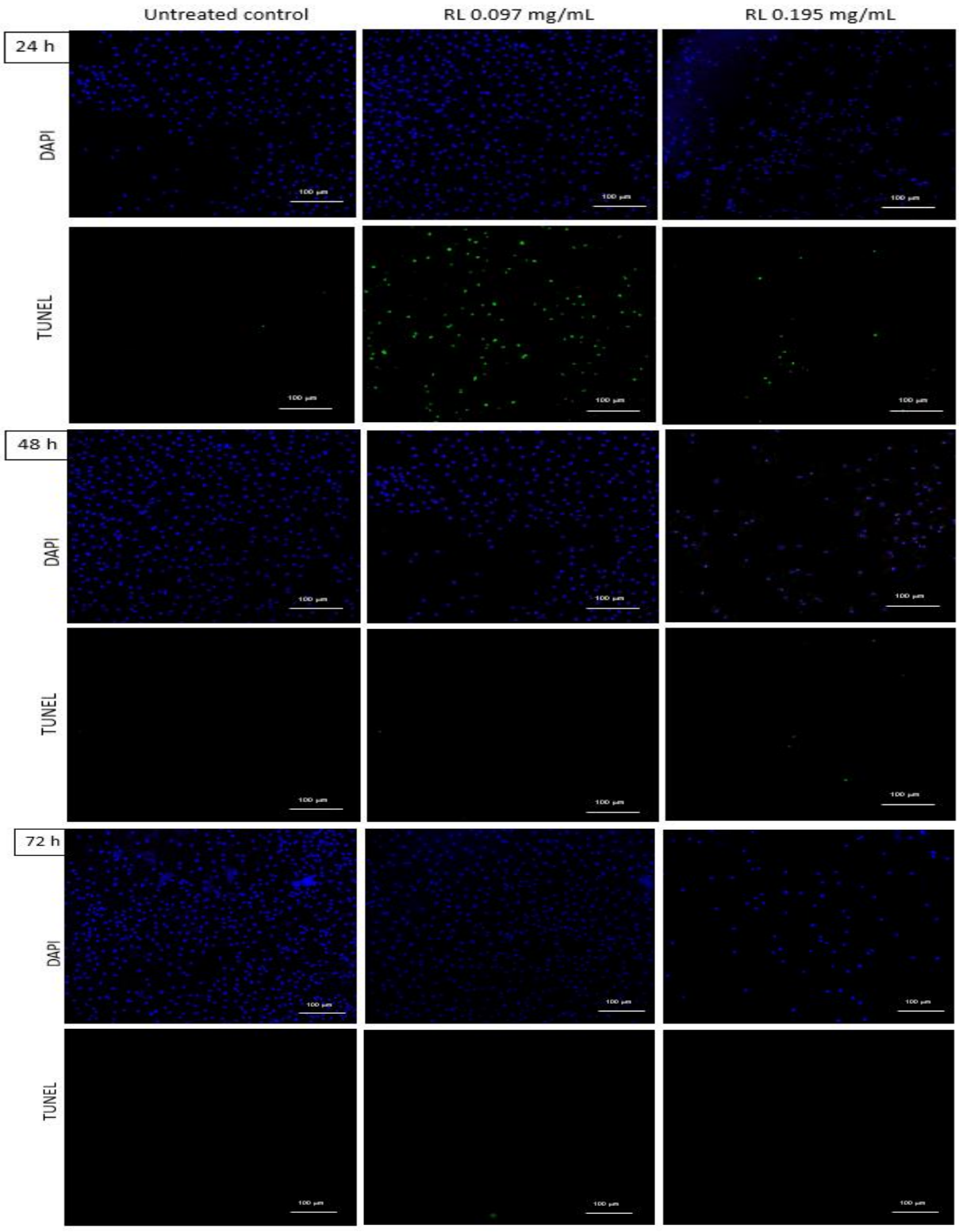


Figure 5.16 **TUNEL assay on rat dental pulp cells treated with rhamnolipid.** The photographs are representative of three independent experiments. RL; rhamnolipid. See appendix II for magnified photomicrographs.

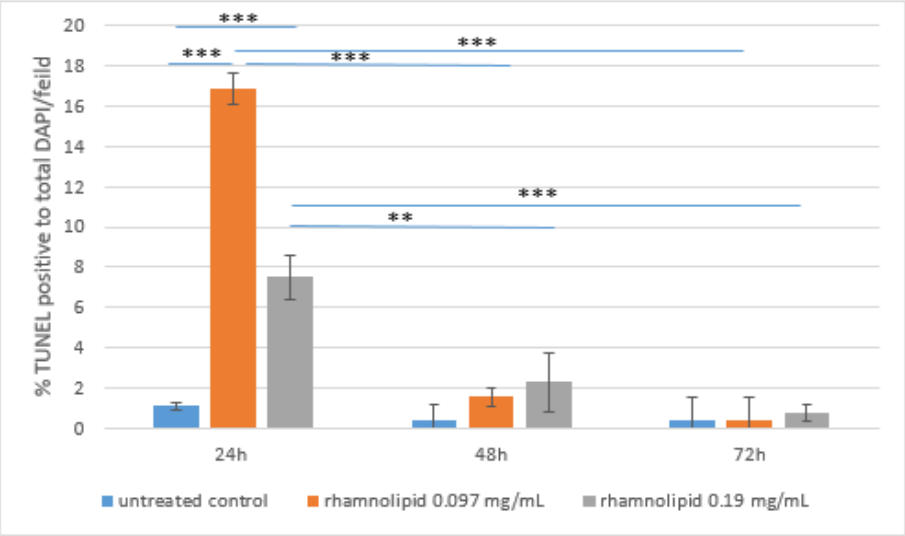


Figure 5.17 **Percentage of apoptotic cells number to the total DAPI-stained nuclei number following treatment with rhamnolipid.** Comparison was made using Tukey's one way ANOVA multiple comparisons test. ***; $p < 0.001$. Error bar represent SEM of three independent experiments.

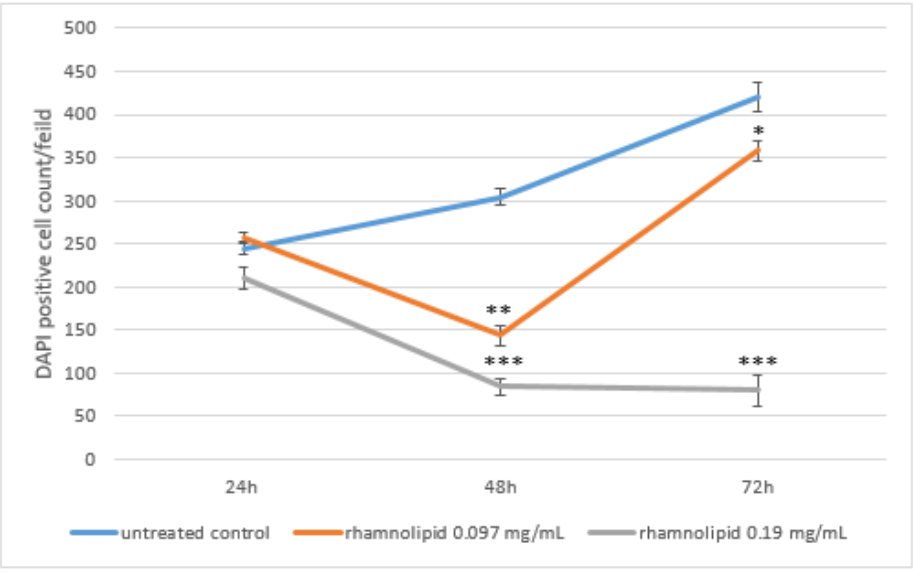


Figure 5.18 **Rat dental pulp cells count following treatment with rhamnolipid for 24, 48 and 72 h.** DAPI-stained nuclei were counted. Comparison was made to the untreated control of each time point using Dunnett's multiple comparisons one way ANOVA test. *; $P<0.05$, **; $P<0.01$, ***; $p< 0.001$. Error bar represent SEM of three independent experiments.

5.3.2.3 RT-qPCR for cytokines

5.3.2.3.1 Inflammatory markers

For further characterisation of rhamnolipid 0.097 mg/mL inflammatory potential on pulp tissue cells, mRNA gene expression of a series of inflammatory cytokines (below) were assayed over 2, 4, 6, 24, 48 and 72 h incubation with 0.097 mg/mL rhamnolipid.

5.3.2.3.1.1 Gene expression profile of pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-6)

5.3.2.3.1.1.1 TNF-alpha

The overall TNF-alpha expression profile (Figure 5.19) suggested a prompt significant ($p < 0.01$) up-regulation by 7.9 fold of the target gene after 2 h incubation of the tooth section with rhamnolipid 0.097 mg/mL relative to the untreated control.

In response to subsequent incubation for 4, 6, 24, 48 and 72 h, TNF-alpha mRNA expression declined significantly ($p < 0.001$). Gene expression dropped from 7.9 folds at 2 h to 2.5 folds at 4 h. At 6 h post incubation, a knocking down of the gene was reported while after 24 and 48 h the gene showed slight but insignificant up-regulation (1.28 and 1.17 folds respectively) to decline again at 72 h (0.59 folds) relative to the control.

5.3.2.3.1.1.2 IL-1beta

A bar chart representation of IL-1beta expression profile is provided in figure 5.19. An up regulation by 3.7 folds of the IL-1beta gene was reported early after 2 h incubation of tooth section with 0.097 mg/mL rhamnolipid ($p < 0.01$) relative to the untreated control sample. Four and 6 h post incubation with the treatment, the pulp tissue expression of IL-1beta mRNA was down regulated significantly ($p < 0.001$). Further incubation of the tooth slice with rhamnolipid solution for 24 and 48 h resulted in insignificant ($p > 0.05$) re-induction of the target gene expression by 2.1 and 2.8 folds respectively which declined again toward the end of the incubation period (72 h) in relation to the untreated control.

5.3.2.3.1.1.3 IL-6

No significant up-regulation of IL-6 mRNA expression was monitored between 2 and 24 h co-culture of the tooth sections with 0.097 mg/mL rhamnolipid solution ($p > 0.05$) (Figure 5.19). At 48 h incubation, a 16.7 folds increase ($p < 0.001$) of IL-6 mRNA expression was reported followed by a decline to 1.9 folds after 72 h relative to the control (Figure 5.19).

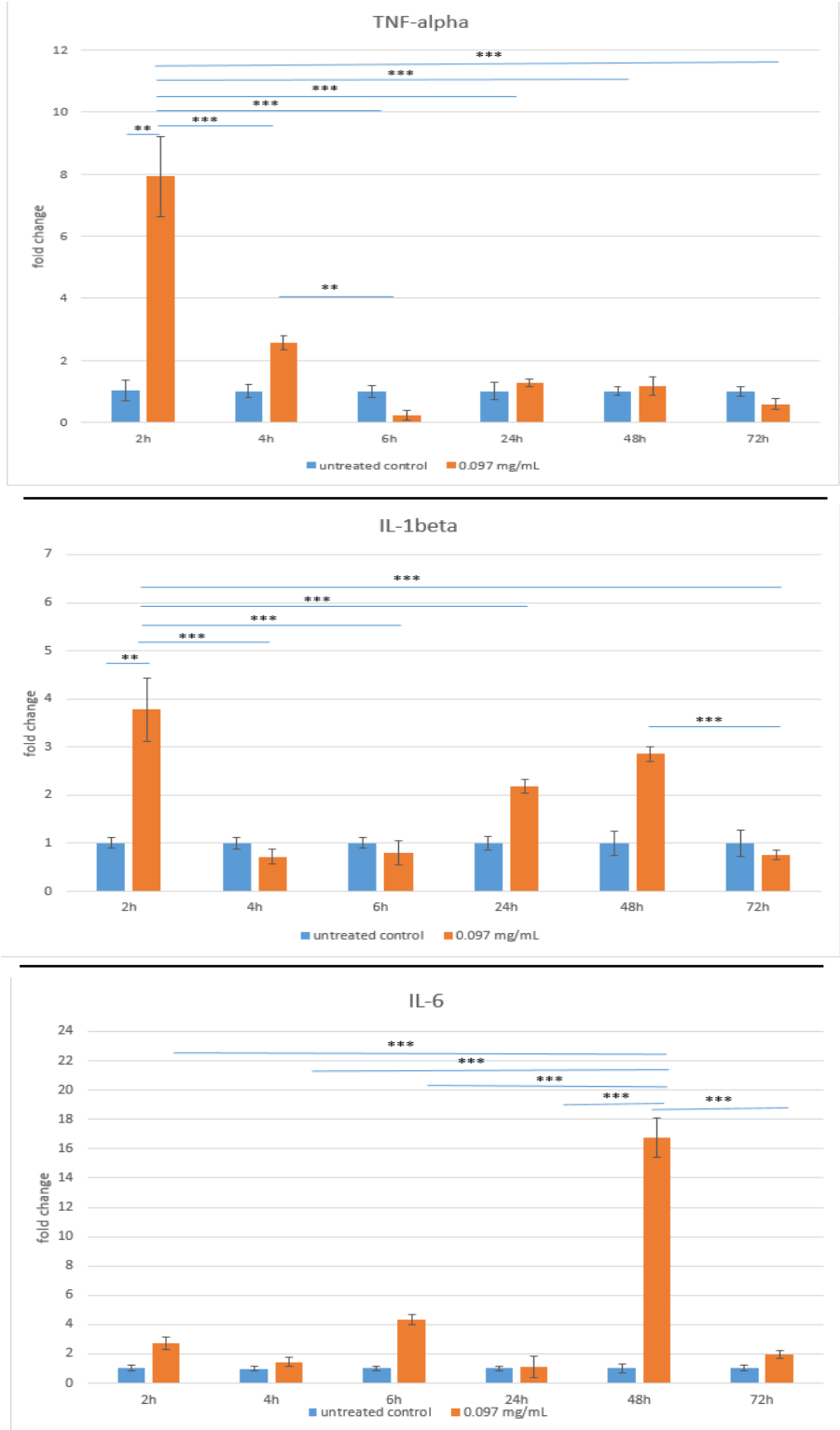


Figure 5.19 **Pro-inflammatory gene expression in response to treatment with rhamnolipid.** Tukey's one way ANOVA was used to determine significant difference among the different groups. *; $P < 0.05$, **; $p < 0.01$, ***; $p < 0.001$. Error bars represent SEM of three independent experiments. Note: B2M was used as the reference gene.

5.3.2.3.1.2 Gene expression profile of anti-inflammatory cytokines (IL-4 and IL-10)

5.3.2.3.1.2.1 IL-4

IL-4 mRNA expression by the cells of the tooth section co-cultured with 0.097 mg/mL rhamnolipid was up-regulated significantly ($p < 0.001$) by 6.2 folds 2 h post incubation (Figure 5.20). A significant ($p < 0.001$) decline in the expression was followed the subsequent time points between 4 and 24 h. After 48 h culture, the gene expression was induced again by 2.1 folds and then knocked down significantly ($p < 0.001$) at 72 h time point (Figure 5.20). The comparison was made relative to the negative control.

5.3.2.3.1.2.2 IL-10

A significant ($p < 0.001$) up-regulation of IL-10 mRNA expression was induced at two time points; 2 and 48 h by 6.5 and 10.9 folds respectively relative to the untreated control (Figure 5.20). At 4, 6 and 24 h the expression declined significantly ($p < 0.001$) by 1.5, 0.8 and 2.2 folds, respectively. Following up-regulation at 48 h, the mRNA expression dropped significantly at 72 h by 1.3 folds (Figure 5.20).

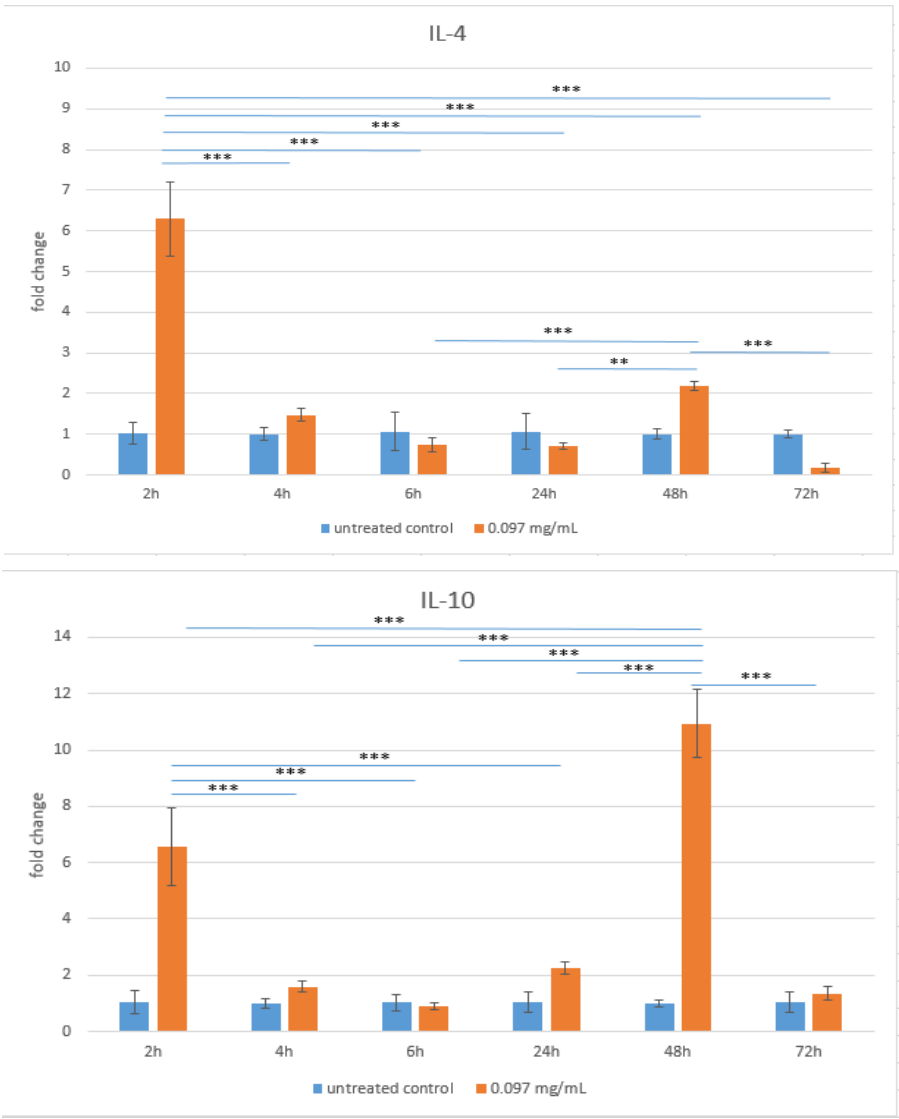


Figure 5.20 **Anti-inflammatory gene expression in response to treatment with rhamnolipid.** Tukey's one way ANOVA was used to determine significant difference among the different groups. *; $P < 0.05$, **; $p < 0.01$, ***; $p < 0.001$. Error bars represent SEM of three independent experiments. Note: B2M was used as the reference gene.

5.3.2.3.1.3 Gene expression profile of TGF-beta1 and beta defensin cytokines

5.3.2.3.1.3.1 TGF-beta1

A slight, but insignificant ($p > 0.05$) up-regulation of the TGF-beta1 mRNA expression was induced only at 2 h post incubation of the tooth section with 0.097 mg/mL rhamnolipid relative to the untreated control (Figure 5.21). Subsequent incubation of the samples with rhamnolipid for 4, 6, 24, 48 and 72 h resulted in down-regulation of the growth factor cytokine mRNA expression which was significant ($p < 0.001$) for the 4, 6 48 and 72 h samples but not for the 24 h sample ($p > 0.05$) when compared to the untreated counterpart controls (Figure 5.21).

5.3.2.3.1.3.2 Defensin beta2

The expression of the antimicrobial peptide defensin beta2 mRNA was up-regulated 2 h post incubation with rhamnolipid by 7.0 fold which was significantly ($p < 0.001$) higher than expression at any other time points (Figure 5.21). At 4 h incubation, the expression was reduced to 4.2 fold and further down regulated the following 6 and 24 h to slightly ($p > 0.05$) up-regulated at 48 h (1.7 fold increase). At the end of the incubation period (72 h), the gene expression was down regulated (0.4 fold) relative to the untreated control (Figure 5.21).

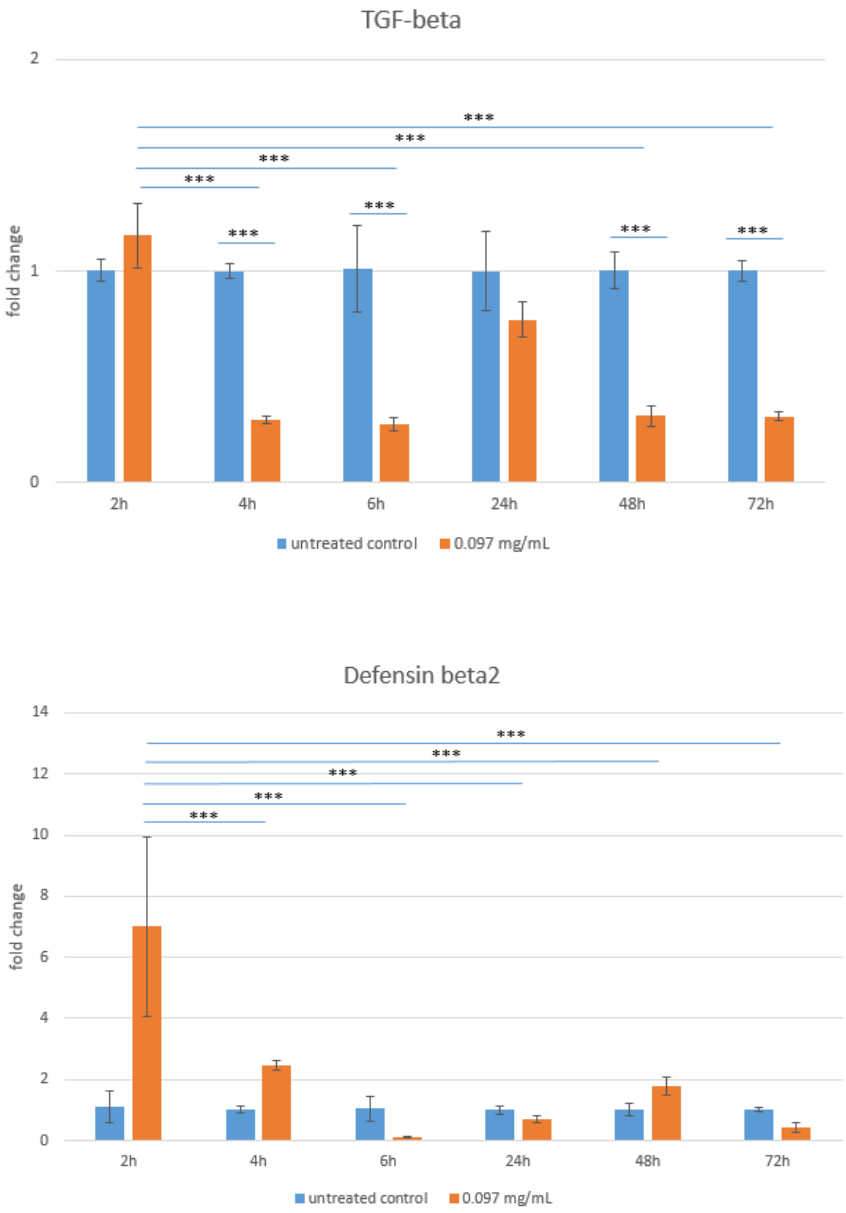


Figure 5.21 **TGF-beta1 and defensin beta2 gene expression in response to treatment with rhamnolipid.** Tukey's one way ANOVA was used to determine significant difference among the different groups. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$. Error bars represent SEM of three independent experiments. Note: B2M was used as the reference gene.

5.3.2.3.2 Apoptotic markers

Treatment with rhamnolipid 0.097 mg/mL for 24 h did not show the potential to induce any of the tested pro- or anti-apoptotic markers (Figure 5.22). Conversely, a down regulation of these markers was the observed trend. However, this down regulation of the apoptotic markers genes expression was not significant ($p > 0.05$) (Figure 5.22).

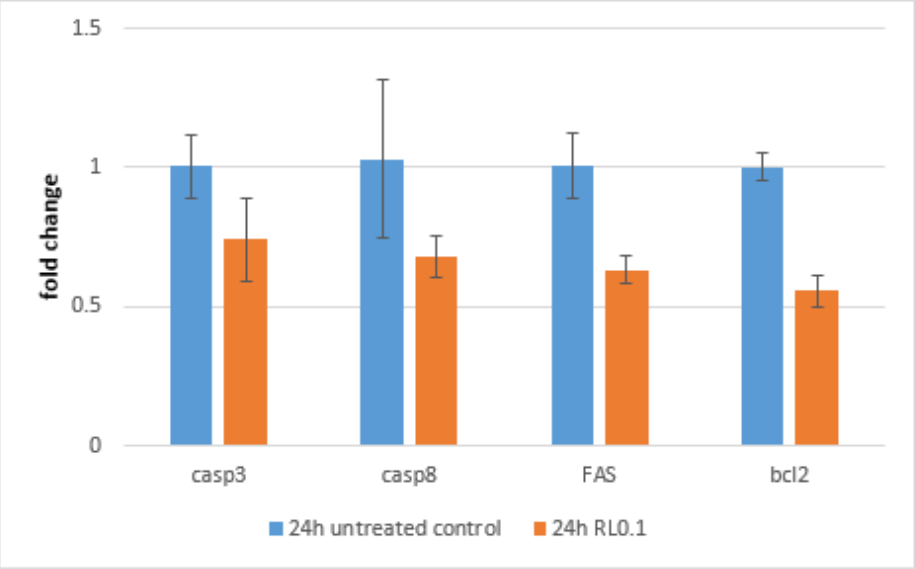


Figure 5.22 **Apoptosis markers mRNA expression in response to treatment with rhamnolipid for 24 h.** Tukey's one way ANOVA was used to determine significant difference among the different groups. Error bars represent SEM of three independent experiments. Note: B2M was used as the reference gene.

5.3.2.4 Bacterial growth under modified culture medium (DMEM + 10% BHI)

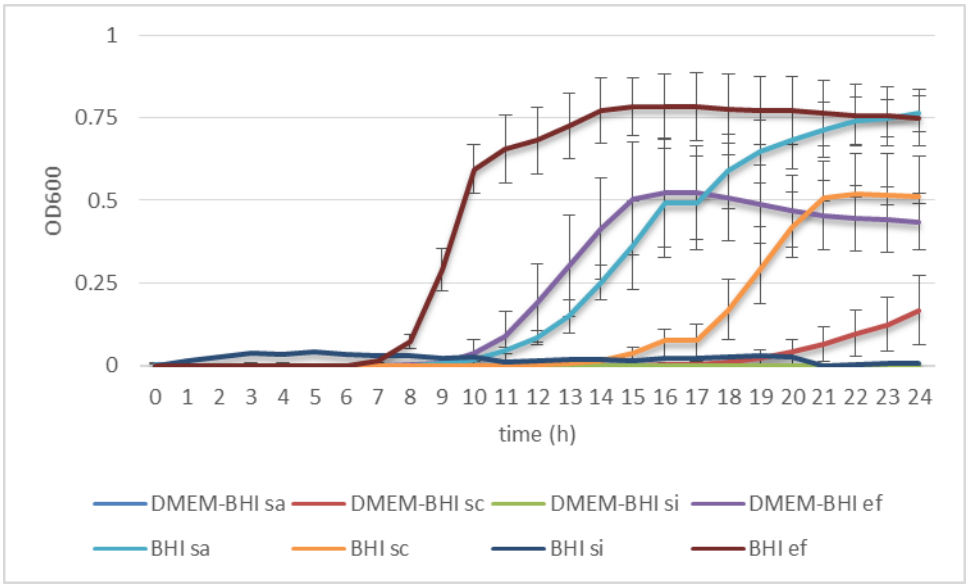
SAG and *E. faecalis* isolates were grown in modified culture medium (DMEM + 10% FBS + 10% BHI) to 10^2 CFU/mL. Figure 5.23a depicts the bacterial growth curves based on the measured absorbance hourly for 24 h as an indication of the change in turbidity relative to bacterial growth. *S. intermedius* did not grow in BHI nor in the modified medium with no measurable growth at 24 h (Figure 5.23a). Similarly, *S. anginosus* was unable to grow in the modified medium. Conversely, it grew in BHI but demonstrated a delayed lag phase (10 h) followed by log phase which continued to the end of the incubation period (24 h). *S. constellatus* growth was further impaired when grown in DMEM + BHI with no measurable growth after 16 h. The maximum absorbance recorded at 600 nm was 0.16 compared to 0.5 when *S. constellatus* grew in BHI medium. *E. faecalis* survived and showed typical growth curve both in BHI and the modified medium. A longer lag phase of 9 h was required for this bacterium before starting the exponential phase when it was cultured in DMEM + BHI than when it grew in BHI (lag phage length = 7 h). Its growth occurred at a slower rate in the modified medium and its maximum absorbance at 600 nm (0.43 ± 0.08) was lower than that in BHI (0.74 ± 0.07) (Figure 5.23a).

Since the tooth section was aimed to be co-cultured with SAG and *E. faecalis* and the rhamnolipid treatment for 8 h and since none of the tested isolates were able to show active growth during the first 8 h of incubation in the modified medium, then a viable cell count of the bacteria was carried out hourly for 8 h as shown in figure 5.23b. *S. intermedius* could be cultured on FAA plates and gave rise to viable colonies only up to 4 h; beyond that no viable cells were recovered. *S. anginosus* cells were cultured on FAA plates and a slight increase in number was evident between zero time ($\log_{10}$ = 1.80 ± 0.12) and 8 h ($\log_{10}$ = 2.40 ± 0.03). Similarly, *S. constellatus* was able to be cultured from the modified medium onto FAA plates with the bacterial yield increased from 1.70 ± 0.15 $\log_{10}$ at zero time to 2.80 ± 0.03 $\log_{10}$ at 8 h. Comparable to its absorbance reading based growth pattern (Figure 5.23a), *E. faecalis* demonstrated viability in the modified medium when cultured on FAA plates. The $\log_{10}$ of the recovered *E. faecalis* increased steadily from 1.20 ± 1.06 at zero time to 5.40 ± 0.04 at 8 h incubation.

However, upon application of this bacterial concentration (10^2 CFU/mL) of *S. anginosus* and *S. constellatus* to the tooth section in the modified culture medium, no supernatant change

in colour was observed, indicative of no growth. No bacterial attachment in the histologically prepared sections was noticed (data not shown). Thus, a higher concentration of the bacterial inoculum (10^6 CFU/mL) were applied to infect the tooth section for 24 h (below).

a-



b-

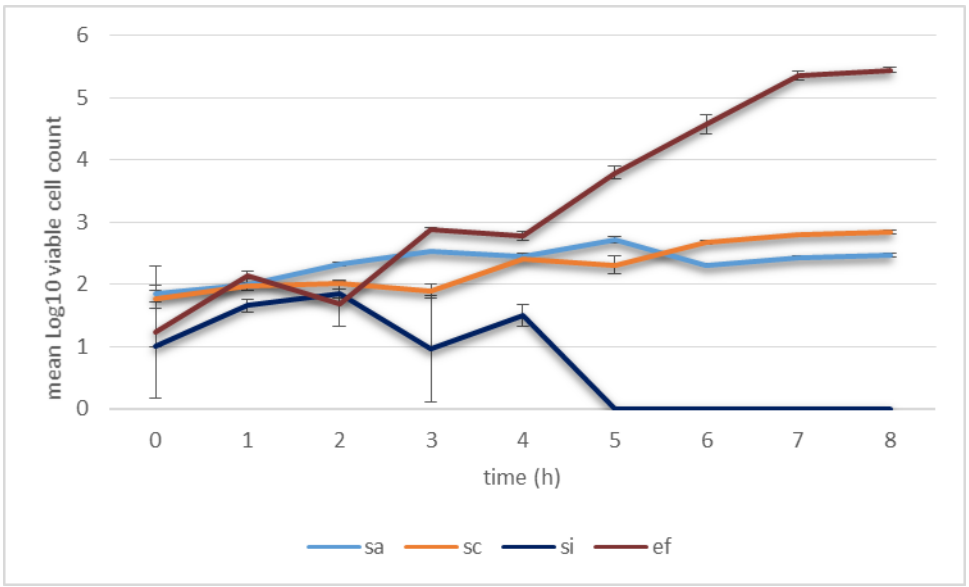


Figure 5.23 **SAG and *E. faecalis* growth and viability in the modified culture medium (DMEM containing 10 % FBS + 10% BHI).** a- Optical density (600 nm) based bacterial growth pattern for 24 h. b- Viable bacterial cell count over 8 h culture in the modified culture medium. Error bars represented the SEM of three independent experiments.

5.3.2.5 *Ex-vivo* rhamnolipid antimicrobial activity

Preliminary experiments (data not shown) involved culturing tooth section with *S. anginosus* or *S. constellatus* (10^6 CFU/mL) and rhamnolipid in serum containing modified culture medium and demonstrated no inhibitory effect of rhamnolipid. The culture supernatants of the infected control and rhamnolipid treatment were turbid and yellowish indicating bacterial growth. Serum inactivation of the rhamnolipid was suggested and a serum-free modified culture medium was employed.

5.3.2.5.1 Histology sections: Gram staining

Sections were treated with modified Gram stain (Brown and Brenn stain), which stains Gram positive bacteria purple (crystal violet), and Gram negative bacteria pink (fuchsin).

Following co-culture of the tooth section with 10^6 *S. constellatus* for 24 h, bacterial cells stained purple were visualised attached to the exposed pulp tissue (Figure 5.24a, red arrows). A zone of cell infiltration (Figure 5.24a, blue arrows) can be seen surrounding the infected area. Subsequent to co-culturing the tooth section with the same bacterial concentration and 0.097 mg/mL rhamnolipid solution, only few *S. constellatus* cocci could be detected in the area between the odontoblasts and the pre-dentine layer (Figure 5.24a, red circles) which might be dead bacterial cells of the infecting inoculum.

Gram staining of the histological sections of tooth slices inoculated with *S. anginosus* at the same concentration (10^6 CFU/mL) for 24 h showed small bacterial aggregates attached to the exposed pulp layer (Figure 5.24b, red arrows). No bacterial invasion deep into the pulp tissue was noticed. Bacterial cocci were also found attached to the periodontal ligament (Figure 5.24b, red arrows). In response to co-culture with rhamnolipid solution at 0.097 mg/mL concentration, only few singular cocci were found attached to the pulp exposed area which might be from the original bacterial inoculum. These findings indicated a successful antimicrobial activity of the rhamnolipid treatment against the two SAG isolates tested.

a- ***S. constellatus***

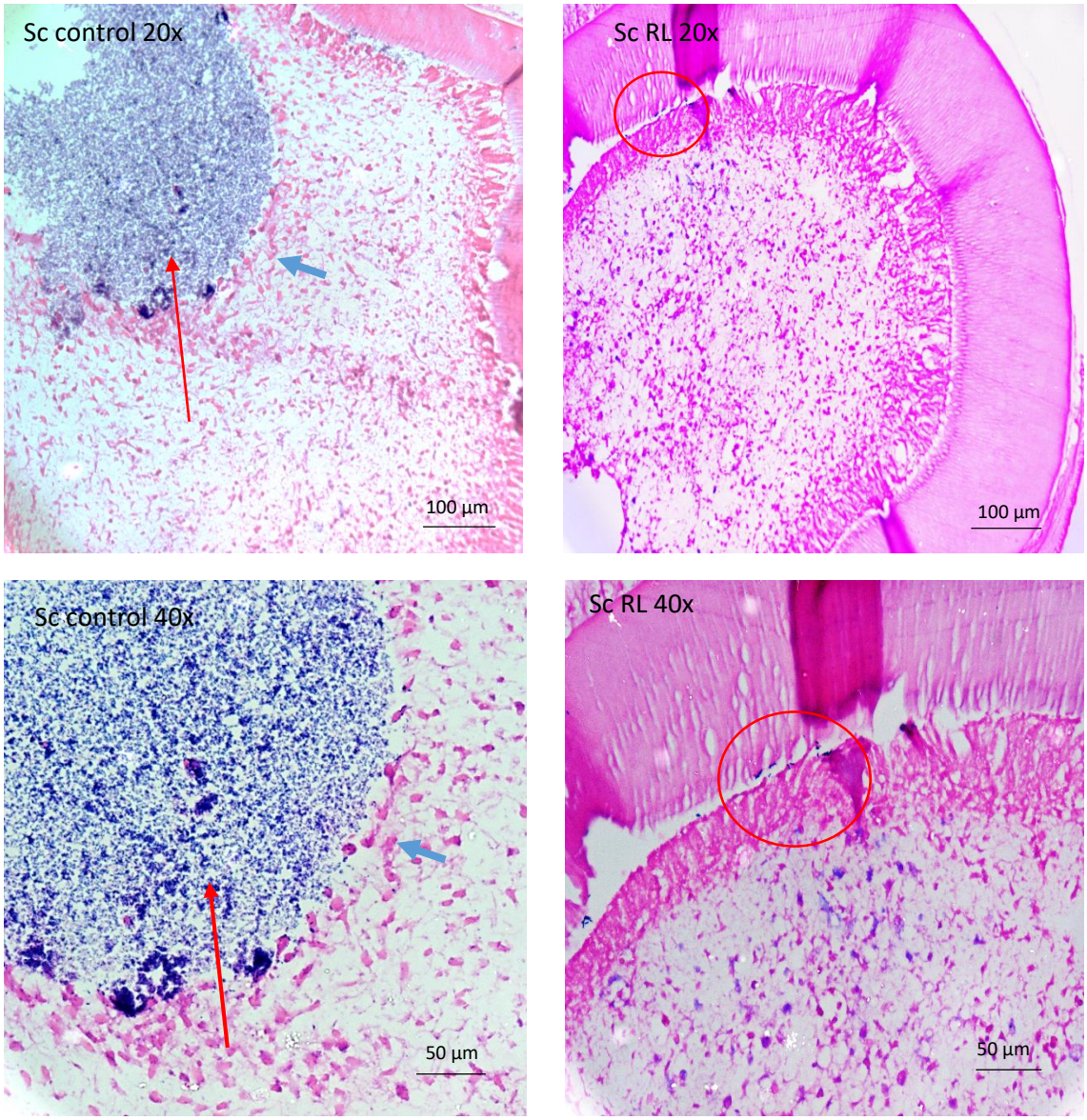
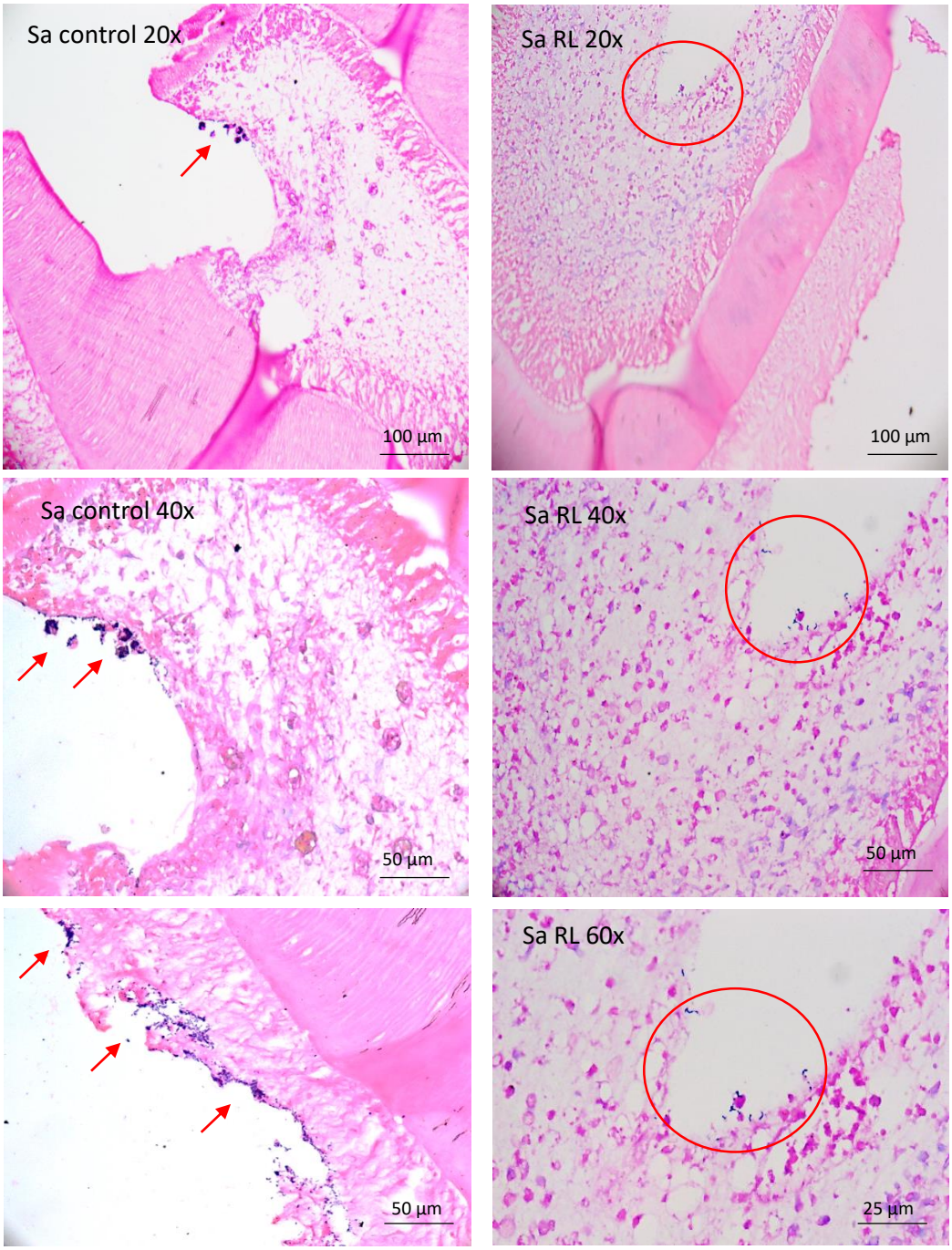


Figure 5.24 ***Ex-vivo* rhamnolipid antimicrobial activity.**

a- *S. constellatus*-infected tooth section was co-cultured with 0.097 mg/mL rhamnolipid for 24 h. Top two microphotographs were taken at 20x magnification. Bottom two microphotographs were taken at 40x magnification. Red arrows represented the Gram stained bacteria and blue arrows represent cell infiltration in the absence of rhamnolipid (negative control sample). Red circles represent the Gram stained bacteria in the presence of rhamnolipid (test sample). The photographs are representative of three independent experiments. Sc; *S. constellatus*, RL; rhamnolipid.

b- ***S. anginosus***



b- *S. anginosus*-infected tooth section co-cultured with 0.097 mg/mL rhamnolipid for 24 h. Top two microphotographs taken at 20x magnification. Middle two microphotographs taken at 40x magnification. Right bottom image taken at 60x magnification. Left bottom micrograph showed bacteria attached to periodontal and surrounding tissue (40x mag). Red arrows represented Gram-stained bacteria in the absence of rhamnolipid (negative control sample). Red circles represent the Gram stained bacteria in the presence of rhamnolipid (test sample). The photographs are representative of three independent experiments. Sa; *S. anginosus*, RL; rhamnolipid.

5.3.2.6 ELISA quantification of cytokines in tissue culture supernatant

5.3.2.6.1 ELISA quantification of pro-inflammatory cytokines

5.3.2.6.1.1 TNF-alpha

ELISA quantification of tooth slice culture supernatant revealed a substantial release of TNF-alpha in all of the treatment groups tested for 24 h in serum free medium (Figure 5.25). A level of more than 300 ng/L TNF-alpha was quantified in the supernatant of the negative control sample. A slightly higher level was detected in the spent culture sample of tooth section treated with the rhamnolipid solution only at 0.097 mg/mL. Tooth sections infected with *S. anginosus* or *S. constellatus* also triggered the release of this inflammatory cytokine at levels (293.5 ± 13.3 ng/L and 317.7 ± 44.2 ng/L respectively) comparable to the control sample (323.0 ± 15.3 ng/L). TNF-alpha quantities released by *S. anginosus* and *S. constellatus*-infected sections treated with rhamnolipid were also in linearity with the untreated negative control (317.4 ± 16.7 and 287.04 ± 13.2 ng/L vs. 323.0 ± 15.3 ng/L respectively). Despite these variations in the levels of TNF-alpha, no statistical significance ($p > 0.05$) was encountered among the different groups using Tukey's one way ANOVA test.

5.3.2.6.1.2 IL-1beta

The levels of IL-1beta cytokine quantified in the supernatant collected for the different tested groups demonstrated significant variations (Figure 5.25). Rhamnolipid-treated tooth section released a significantly higher level (93.2 ± 25.4 pg/mL) of IL-1beta than the untreated negative control (59.2 ± 5.6 pg/mL; $p < 0.05$) and the bacteria infected samples (37 ± 3.1 pg/mL by *S. anginosus* infected sample; $p < 0.001$ and 47.2 ± 11.3 pg/mL by *S. constellatus* infected sample; $p < 0.01$). Subsequent to the co-culture of the tooth section with *S. anginosus* and rhamnolipid for 24 h, a strikingly high level of the target inflammatory protein was excreted (141.2 ± 19.7 pg/mL) which was significantly higher ($p < 0.001$) than the pathogen-infected tooth section alone. Similarly, though not significant ($p > 0.05$), an enhanced release of IL-1beta (68.9 ± 3.2 pg/mL) was reported for *S. constellatus*-infected tooth section co-cultured with rhamnolipid for 24 h when compared to *S. constellatus*-infected sample.

5.3.2.6.1.3 IL-6

The release of IL-6 (Figure 5.25) followed the same trend as TNF-alpha. No statistical significant variation ($p > 0.05$) in the levels of IL-6 was measured among the different groups assayed. *S. anginosus*-infected tooth section demonstrated the highest level of excreted IL-6 (20.0 ± 1.7 ng/L). Rhamnolipid only treated tooth section released the least quantity of this inflammatory cytokine (15.2 ± 1.9 ng/L) into the culture supernatant. A lower level of IL-6 was detected for sections co-cultured with the *S. anginosus* isolate and rhamnolipid (16.5 ± 2.1ng/L) than for sections co-incubated with the pathogen alone (20.09 ± 1.7 ng/L). Similarly, *S. constellatus* infected sample treated with rhamnolipid was found to release less IL-6 (17.2 ± 1.7 ng/L) than the infected sample with no rhamnolipid treatment (19.01 ± 1.09 ng/L) but the difference was insignificant ($p > 0.05$).

5.3.2.6.1.4 IL-18

The release of the pro-inflammatory protein IL-18 into the culture supernatant did not show any significant difference ($p > 0.05$) among the tested groups (Figure 5.25). Similar to IL-6, IL-18 was produced at the least level (178.6 ± 12.4 pg/mL) by rhamnolipid-treated tooth slice in comparison to other groups. Other treated group samples excreted comparable levels of the tested cytokine into their spent culture supernatant ($p > 0.05$).

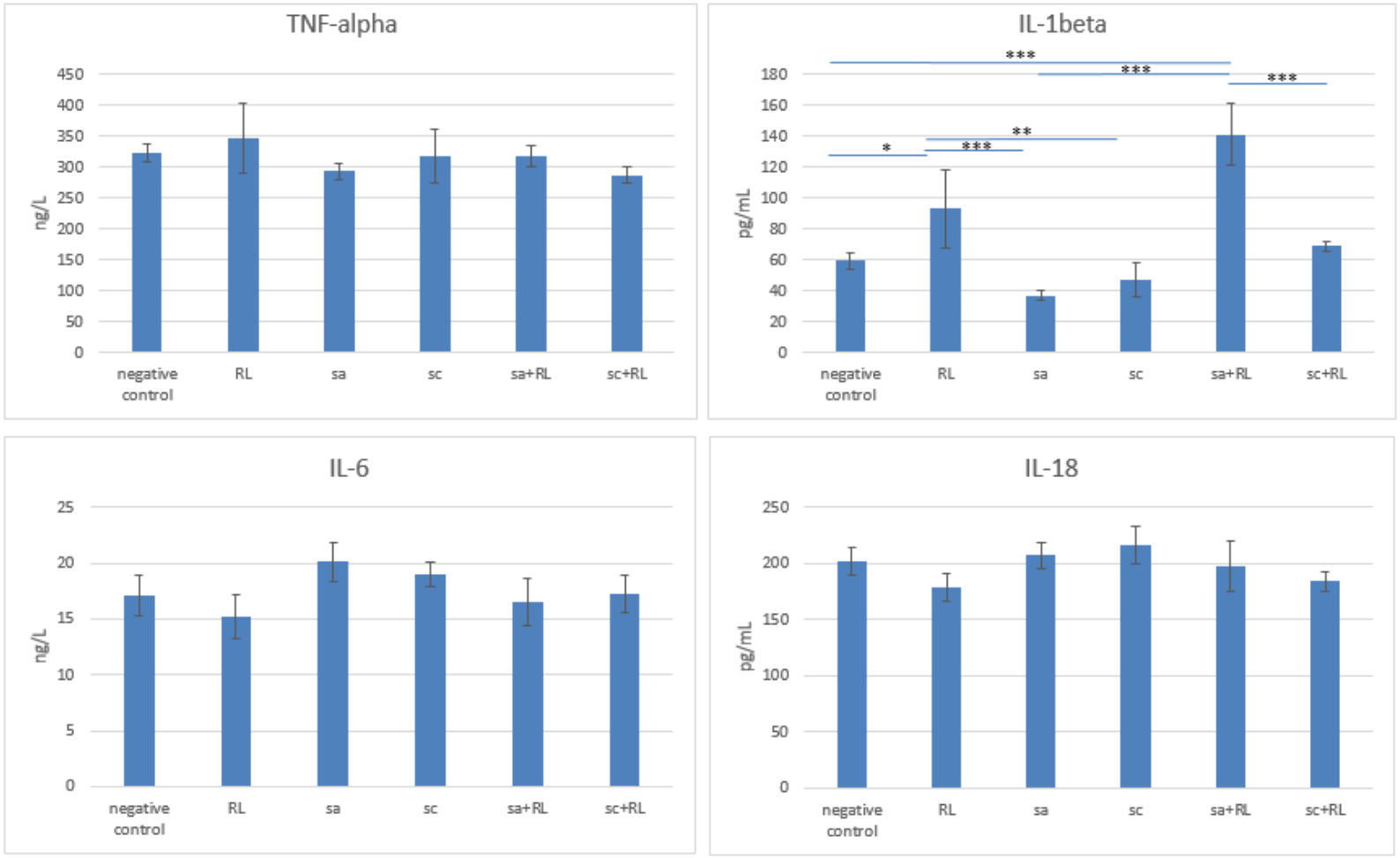


Figure 5.25 **Pattern of pro-inflammatory cytokines release from infected tooth section treated with rhamnolipid for 24 h.** Bar charts represent TNF-alpha, IL-1beta, IL-6 and IL-18 levels in the culture medium of bacterially infected tooth sections treated with rhamnolipid 0.097 mg/mL by ELISA. Error bar represents SEM of three independent experiments. *; $p < 0.05$, **; $p < 0.01$ and ***; $p < 0.001$ using Tukey's one way ANOVA. RL; rhamnolipid, sa; *S. anginosus*, sc; *S. constellatus*.

5.3.2.6.2 ELISA quantification of anti-inflammatory cytokine IL-10, transforming growth factor TGF-beta and antimicrobial peptide defensin-beta2

5.3.2.6.2.1 IL-10

The collective data obtained from ELISA assay result did not show large variations in the IL-10 levels among the different treatment groups (Figure 5.26). Rhamnolipid treated sample supernatant released the least quantity of IL-10 (174.5 ± 22.0 pg/mL) which was significantly ($p < 0.05$) lower than the untreated negative control. *S. anginosus* infected sample supernatant was found to contain comparable amount of IL-10 to that of the negative control and the rhamnolipid treated infected sample supernatants ($p > 0.05$). *S. constellatus*-treated sample produced significantly higher quantity of IL-10 than that produced by the rhamnolipid treated sample ($p < 0.05$).

5.3.2.6.2.2 TGF-beta

The pattern of TGF-beta release into the culture supernatant did not express significant variation among the different treated groups (Figure 5.26). Rhamnolipid treated tooth section was found to release the lowest quantity of the growth factor into the supernatant (477.1 ± 61.5 ng/L) when compared to the other group ($p > 0.05$). Although not significant ($p > 0.05$), rhamnolipid treated *S. anginosus* infected sample showed a slight increase of TGF-beta release compared to the *S. anginosus* infected sample (677.1 ± 50.5 vs. 629.3 ± 37.1 ng/L, respectively). Conversely, tooth section cultured with *S. constellatus* alone produced a higher level of TGF-beta than the section co-cultured with *S. constellatus* and rhamnolipid (676.3 ± 68.09 ng/L vs. 608.2 ± 78.2 ng/L respectively). However, this difference was not significant ($p > 0.05$).

5.3.2.6.2.3 Defensin-beta2

The profile of defensin-beta2 excretion into the culture medium demonstrated significant variation among the different treatment groups collected 24 h post incubation (Figure 5.26). ELISA assay revealed that the negative control sample produced the highest level of defensin-beta2 (198.2 ± 78.1 ng/L) which was statistically significantly ($p < 0.01$) higher than other treatment groups except that of the *S. anginosus*-treated tooth section ($p > 0.05$). Treating *S.*

anginosus and *S. constellatus*-infected tooth sections with rhamnolipid 0.097 mg/mL caused a significant ($p < 0.01$) reduction of the level of the excreted densin-beta2 released into the medium when compared to the untreated negative control.

Interestingly, *S. anginosus* infected tooth section demonstrated the ability to release significantly ($p < 0.01$) higher quantity (166.5 ± 30.9 ng/L) of the antimicrobial peptide into the culture medium than the pathogen infected tooth section treated with rhamnolipid (14.6 ± 5.6 ng/L). No significant difference ($p > 0.05$) of the peptide levels in the culture medium collected was calculated between *S. constellatus* infected tooth slices and their counterparts treated with rhamnolipid.

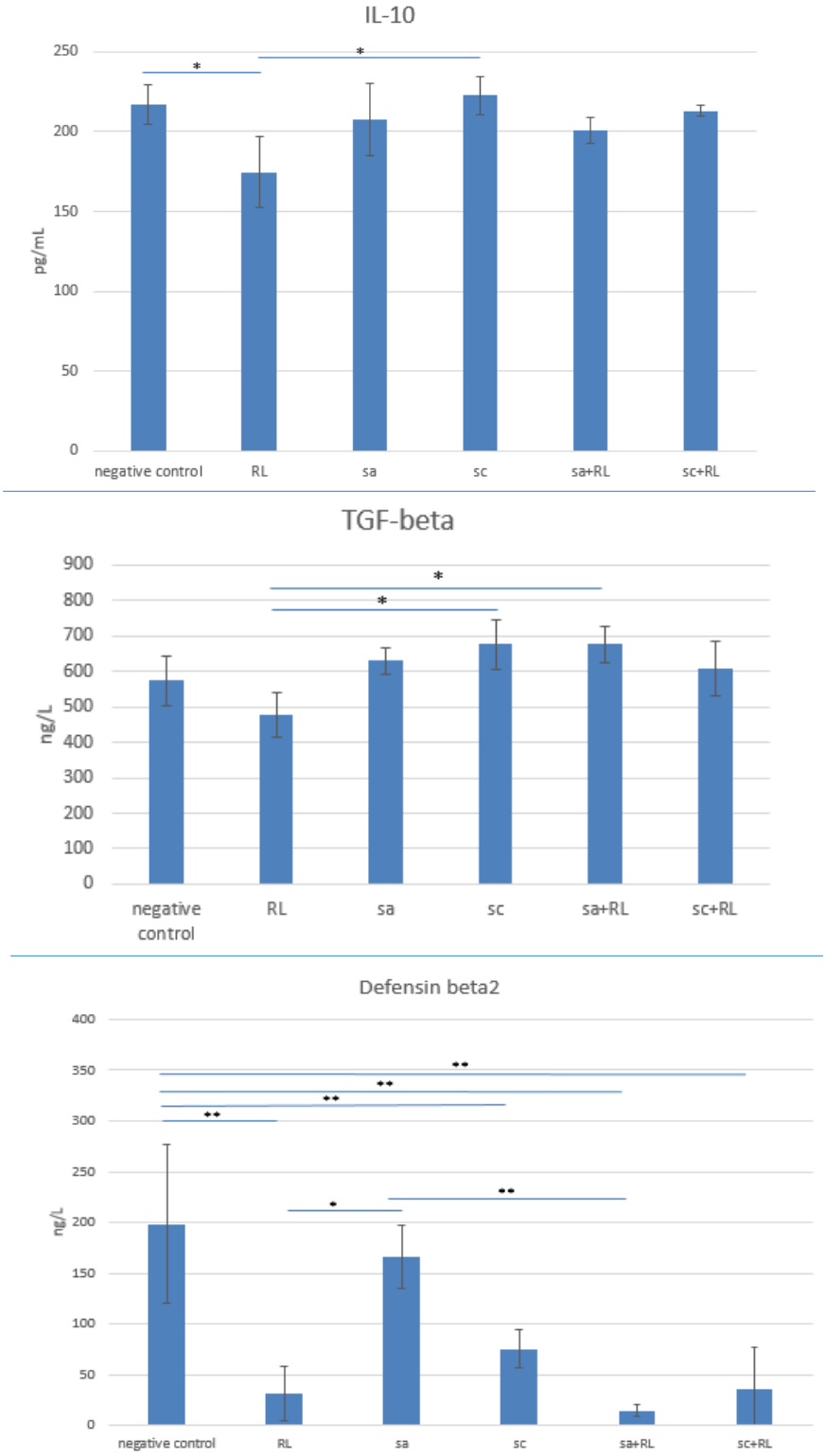


Figure 5.26 **Pattern of anti-inflammatory cytokine** IL-10, transforming growth factor TGF-beta and antimicrobial peptide defensin-beta2 release from tooth section treated with rhamnolipid for 24 h. Bar charts represent IL-10, TGF-beta and Defensin beta2 levels in the culture media of bacterially infected tooth sections treated with rhamnolipid 0.097 mg/mL by ELISA. Error bar represents SEM of three independent experiments. *; $p < 0.05$, **; $p < 0.01$ and ***; $p < 0.001$ using Tukey's one way ANOVA. RL; rhamnolipid, sa; *S. anginosus*, sc; *S. constellatus.*

5.3.2.7 Serum effect on rhamnolipid activity on pulp cells

Cells that were cultured in 0 % FBS αMEM medium for 24 h did not grow properly as only 2-3 cells were counted per field (Figure 5.27a). In the presence of 2-20 % FBS, cells were able to grow and demonstrated normal morphology (Figure 5.27a). Treating cells that had been growing in medium containing 0-5 % FBS with 0.097 mg/mL rhamnolipid resulted in a significant toxicity (Figure 5.28a) indicated by few dead cells remaining attached to the well surface that stained blue with trypan blue (Figure 5.27a). At 10 and 20 % FBS, introducing rhamnolipid treatment did not cause toxic effect on cells and showed no difference ($p > 0.05$) to the untreated control cells (Figure 5.27a and 5.28a).

With cells were grown in 2-20 % FEB for the first 24 h and then in 0 % FBS for the second 24 h (Figure 5.27b), rhamnolipid treatment during the second 24 h caused significant cytotoxic effect on cells in comparison to the untreated control in all serum concentrations tested (Figure 5.28b) indicating a negligible residual serum effect on rhamnolipid activity.

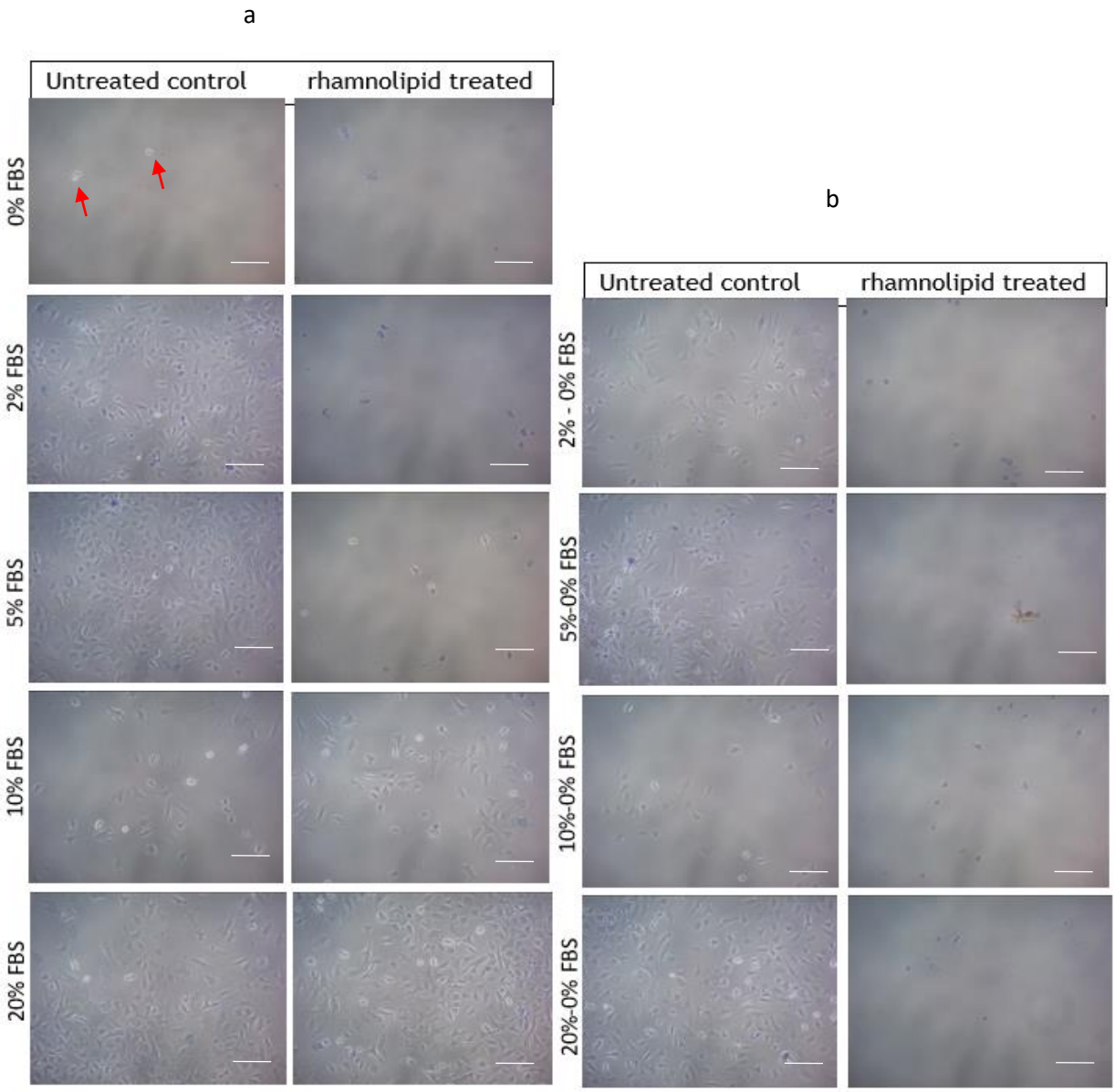


Figure 5.27 **Effect of serum on rhamnolipid cytotoxicity.** Cells were grown for 24 h in αMEM medium containing different concentrations of FBS (0-20%) then divided into two sets. One set was cultured for 24 h in the same concentration of FBS **(a)** and the other set cultured for the second 24 h in medium containing 0 % FBS **(b).** The two groups were then treated with rhamnolipid 0.097 mg/mL. The microphotographs (10x magnification) are representative of three independent experiments. Scale bar=100µm.

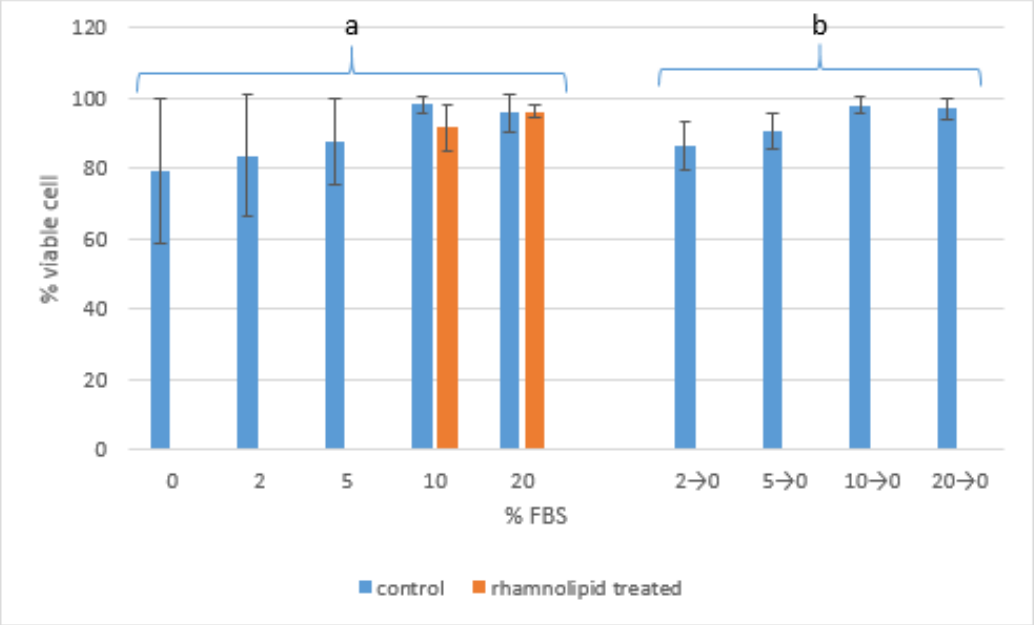


Figure 5.28 **Percentage of viable pulp cells treated with 0.097 mg/mL at different serum concentrations.** The Serum concentration between 0 and 5 % caused 100% toxicity of pulp cells while 10 % and 20 % FBS inactivated rhamnolipid inhibiting its cytotoxic effect **(a).** Residual serum was found to have no effect on rhamnolipid activity indicated by cell toxicity **(b).** Error bars represent SEM of three independent experiments.

5.4 Discussion

Preserving vital pulp tissue and minimising the need for root canal therapy is gaining increasing importance in contemporary endodontics. One of the most crucial steps of successful regenerative endodontic is the complete elimination of residual infection (Albuquerque *et al.* 2014; Kamocki *et al.* 2015).

The current chapter aimed to assess the applicability of *Lp*-BS and rhamnolipid as pulp antimicrobial agents in an *ex-vivo* relevant setting employing a previously developed and characterised rodent dental model (Roberts *et al.* 2013). Biosurfactant toxicity and immunogenicity to the pulp tissue cells were also investigated.

5.4.1 *Lp*-BS

The data obtained suggests that *Lp*-BS at 10 mg/mL was significantly toxic to the pulp cells and tissue. This was evident by the significantly lower count of cells treated with the biosurfactant and lower mRNA yield collected from tooth sections co-cultured with the biosurfactant (Figure 5.10). This toxicity might impact the obtained immune response by causing death to certain cells that contribute in pulp immunity. Down regulation of the expression of most of the tested inflammatory, growth factor and antimicrobial peptide genes in tissue treated with 10 mg/mL *Lp*-BS in comparison to tissue treated with the non-toxic 0.625 mg/mL *Lp*-BS might be related to such toxicity (Figures 5.11-5.13). Although it has been previously suggested that dental pulp can tolerate the toxic effect of restorative materials if bacteria and/or their toxins can be omitted from the pulp, the failure rate of the pulp therapy reported (Akhlaghi and Khademi 2015) might indeed be due to such restorative materials toxicity. Therefore, because of its toxicity and likelihood to account for failure, *Lp*-BS might be inappropriate to be used in pulp therapy. Thus, a decision of not to precede with further investigating the crude *Lp*-BS application to the *ex-vivo* model was made. The successfully partially purified fraction devoid of the contaminants of *Lp*-BS achieved by FPLC (Chapter 3) might show less or no toxicity and thus could be assessed for such application in the future.

Although apoptosis-induced toxicity was reported for *Lp*-BS, demonstrated by TUNEL assay positivity, cell morphology and apoptotic markers gene expression up-regulation, this property has been reported advantageous for a number of biosurfactants as antitumor agents. This opens future prospects for possible investigation of *Lp*-BS as antitumor agent.

For instance, lipopeptide biosurfactants produced by *Bacillus subtilis* (Kim *et al.* 2007) and *Bacillus natto* T-2 (Wang *et al.* 2007) have been proposed as promising antitumor agents via induction of apoptotic effect on LoVo cells and human leukaemia K562 cells respectively. *Candida antarctica*-derived glycolipids biosurfactants have also been reported to contribute to growth arrest, apoptosis, and differentiation of mouse malignant melanoma and human HL60 cells (Sudo *et al.* 2000; Zhao *et al.* 2000). The molecular mechanism of biosurfactant-induced apoptosis is still not well clarified. An increase in Ca^{2+} which evoked Extracellular Signal-regulated Kinases (ERK) phosphorylation has been proposed as a possible mechanism of *Bacillus natto T-2*-derived cyclic lipopeptide biosurfactant pro-apoptotic activity on K562 cells (Wang *et al.* 2009). This ERK phosphorylation subsequently activated Bax, cytochrome c and caspase-3 leading to apoptosis in K562 cells. A membrane permeabilisation of melanoma A375 cells was proposed as a different mechanism of the lipopeptide biosurfactant pseudofactin II (PFII) pro-apoptotic activity (Janek *et al.* 2013). The interaction of PFII micelles with the plasma membrane impaired membrane integrity, evident by the release of LDH, leading to the observed Ca^{2+} influx which triggers further signalling cascades, like measured caspase-3 activation ending up with cell apoptosis.

A concern that needs to be mention here is that *Lp*-BS may form an adhesive layer on top of the exposed ends of the tooth sections. In this case it might be that this adhesive layer prevented proper oxygenation of the pulp cells and thus resulted in cells toxicity.

5.4.2 Rhamnolipid

Rhamnolipid 0.097 mg/mL was established to exert the potential to inhibit *in-vitro* SAG growth (Chapter 4), thus a number of assessments were performed in this chapter to analyse its cytotoxic and immunogenic effects on pulp cells and tissue. An assessment of bacterial growth in the modified serum-containing tissue culture medium (DMEM+10 % FBS + 10% BHI) was also performed. *S. anginosus* and *S. constellatus* survived in the modified medium and thus were selected to infect the tissue to assess the antimicrobial activity of rhamnolipid. However, subsequent preliminary experiment of co-culturing *S. anginosus* and *S. constellatus*-infected tooth sections with rhamnolipid 0.097 mg/mL in FBS-containing modified medium demonstrated no bacterial inhibitory effect of rhamnolipid. The lack of rhamnolipid antimicrobial effect might suggest inactivation of the biosurfactant antimicrobial effect

possibly via binding to serum protein/s. Therefore, serum-free culture medium was employed and as a result, rhamnolipid was shown to exert antimicrobial activity in this medium.

Consequently, all results of rhamnolipid in serum-containing medium including viability assay, TUNEL assay for apoptosis and genes expression of inflammatory, TGF-beta1 and defensin-beta2 might be unreliable. Moreover, it might be that the observed cytotoxicity and immunogenicity of the currently tested rhamnolipid on pulp cells and tissue was due to the unbound rhamnolipid molecules or to the 10 % contaminants of the 90 % pure commercial rhamnolipid used. Therefore, these findings are difficult to interpret with confidence.

Earlier studies mainly focused on the serum effect on rhamnolipid cytotoxicity. To the best of our knowledge, there is no study discussing the serum effect on antimicrobial activity of rhamnolipid. Rhamnolipid could bind with high affinity to serum albumin at 1:1 to 1:2 ratio (Sánchez *et al.* 2008). Therefore, the concentration of free rhamnolipid, due to protein binding, might be reduced to a level below the MIC for SAG resulting in no antimicrobial effect.

The presence of serum in DMEM culture medium severely attenuated rhamnolipid cytotoxicity on dental pulp cells particularly at concentration of ≥ 10 % FBS (Figure 5.27 and 5.28). This finding correlated with previously reported findings of rhamnolipid effects on macrophage cells (McClure and Schiller 1992; Häussler *et al.* 1998) and fibroblast cells (Stipcevic *et al*. 2005).

It has been shown that the presence of FBS severely reduced rhamnolipid cytotoxicity (McClure and Schiller 1992; Häussler *et al.* 1998; Stipcevic *et al.* 2005). An interesting study conducted by Jian and colleagues (2014) reinvestigated the selective rhamnolipid antitumor effect against cancerous but not normal cells. Rhamnolipid was found to exert cytotoxic effect equally against cancer and normal cells due to a reduction in surface tension of the culture medium. The authors demonstrated that the presence of FBS in the culture medium significantly diminished rhamnolipid cytotoxicity which was proposed to be due to the inhibited surface activity. These findings corrected the misleading previous claim that rhamnolipid selectively kills cancer cells which was incorrectly made due to the error in experimental design in which cancer cells were cultured in the absence of serum while normal cells were grown in serum-containing medium (Thanomsub *et al.* 2006).

Rhamnolipids have been well accepted for *in-vivo* animal safety (Federal Register, 2004), though demonstrating cytotoxicity in serum-free medium. This safety can be due to serum-protein binding (Jiang *et al.* 2014). On the other hand, serum protein binding can be reversible and the bound portion may behave as a depot or reservoir from which a continuous slow release of the drug can be maintained (Banker and Clark 2008). Further work need to be performed to utilise this propensity of rhamnolipid serum protein binding by adjusting the concentration for a controlled release pharmaceutical form.

Although *S. constellatus* and *S. anginosus* were applied to infect the tooth section at the same concentration and although the two strains showed comparable *in-vitro* attachment and biofilm potential on plastic well surface (Chapter 2), the former was shown to have a higher affinity to bind to the tooth soft tissue, mainly to the pulp (Figure 5.24a). This highlighted the importance of such *ex-vivo* tooth model to further understand the bacterial attachment in a more relevant situation. *S. anginosus* demonstrated a higher affinity to adhere to the remnant of the periodontal ligament region and to a lesser extent to the exposed pulp tissue (Figure 5.24b). However no statistical analysis on this observation was possible and estimation was done based only on visual basis. This difference in the pulpal affinity and possible infectivity of the two species belonging to the same SAG group sheds light on the importance of bacterial identification to the species level. Little information is available considering the differences in SAG members' pathogenicity and adherence. In a study published 2011 (Japanese Society of Chemotherapy Committee on guidelines for treatment of anaerobic infections and Japanese Association for Anaerobic Infection Research 2011), among 39 *Streptococcus* spp. isolated from dental infection, 16 strains were found to be *S. constellatus* (35.6 %) while only 2 strains were identified as *S. anginosus* (4.4 %). More recently, *S. constellatus* of the SAG group was identified among the most abundant microorganisms, found in highest numbers within individual dental abscesses (George *et al.* 2016). Interestingly, higher adherence to the PGs decorin and biglycan was generally noted for *S. constellatus* isolates over *S. anginosus* isolates (Landrygan-Bakri *et al.* 2012) which might explain the higher adherence potential of *S. constellatus* found herein.

Hydrolytic enzymes are generally considered to be potential virulence factors (Homer *et al.* 1993). In screening for a number of hydrolytic enzymes of SAG isolates in relation to infection, Jacobs and Stobberingh (1995) documented that a number of these enzymes were found

prominent in *S. constellatus* and *S. intermedius* strains but not in *S. anginosus*. Specifically, among the three species of 518 SAG strains, ribonuclease activity was equally dispersed while hyaluronidase, deoxyribonuclease and chondroitin sulphatase activity was found to be allied mainly to *S. intermedius* and *S. constellatus* and was associated with infection-related strains (Jacobs and Stobberingh 1995). Based on these findings, it could be suggested that *S. constellatus* may bear a more potential ability for initiating dental infection. Yet, further investigation on a wide range of SAG species need to be done before uttering any conclusive statement.

Although a high concentration of bacterial inoculum (10^6 CFU/mL) was applied to infect the tooth section for 24 h, no difference in tissue structure was noticed in comparison to the untreated control. Conversely, in a study conducted earlier (Roberts *et al.* 2013) employing the same rodent 3D-tooth model, a different infection pattern of SAG members was reported. The bacterial isolates inoculated at lower concentration of 10^2 CFU/mL were shown able to adhere directly to the central region of the pulpal matrix in small foci that were associated with a localised matrix breakdown. The different colonisation style obtained in this study can be due to the use of different strains of SAG isolates (*S. constellatus* 45386 and *S. anginosus* 39/2/14A) and that visualising bacterial attachment was made by FDA fluorescent stain instead of Gram's stain. FDA stain might be subjective to interpretation error and diffusion and artifacts especially with auto-fluorescing tissue like the tooth tissues taking into account the authors did not provide an FDA-stained bacterial positive control. Moreover, pre-culturing the tooth sections for 5 days prior to co-culturing with bacterial isolates (Roberts *et al.* 2013) might be an additional factor affected pulp tissue status making it weaker and more liable to the damaging effect of bacteria especially if it involved immune cells (Morotomi *et al.* 2019).

In mammals, microbial associated molecular peptide (MAMP) sensitivity leads to the induction of the inflammatory response with the production of cytokines including interleukins and the tumour necrosis factor-alpha (TNF-alpha). Years ago, lipopeptides were shown to induce human innate immune responses through the pattern recognition receptor (PRR) Toll-like receptor TLR2 perception, by activating the transcriptional activator of multiple host defense genes NFkB, the production of interleukin (IL)-12 and the respiratory burst (Hauschildt *et al.* 1990; Aliprantis *et al.* 1999; Brightbill *et al.* 1999; Takeuchi *et al.* 2000; Gerold *et al.* 2008). Recently, it has been shown that rhamnolipids are involved in eliciting

animal defense responses and, like lipopeptides, can be thus classified as MAMPs (Vatsa *et al.* 2010). Rhamnolipids can play dual role: as antimicrobial and also can stimulate host defense immunity. This dual property is probably very important for the efficacy of new therapy. However, because a wide range of rhamnolipids congeners and homologues have been isolated and characterised, the biological effects obtained have been variable as well (Abdel-Mawgoud *et al.* 2010).

Interestingly, all the markers tested in the current study by ELISA were detected in all the samples assayed including the untreated control and the infected sections with and without rhamnolipid treatment. This might be due to tissue response to the process of extraction and cutting, though the sections were pre-cultured for 24 h to allow for amelioration of such response.

Among the four pro-inflammatory markers monitored 24 h post rhamnolipid application with the infected tooth section, only IL-1beta was at a significantly higher level in rhamnolipid treated sections (control or infected sections) in comparison to the uninfected or bacterially infected tooth sections where it was also detected but at lower levels. This finding is promising as it might indicate a moderate immune response to rhamnolipid treatment. Moderate inflammation is an expected first step of pulp tissue repair cascade in response to direct pulp capping followed by the commitment of adult reserve stem cells, their proliferation and terminal differentiation. For many years, inflammation in pulp healing has been considered as undesirable effect which can lead to necrosis. However, recent results highlighted the potentially beneficial impact of moderate balanced inflammation on tissue healing and regeneration (Goldberg *et al.* 2008), in part, through stimulating progenitor cells. Inflammation has been shown to appear 3-7 days after implantation of pulp capping molecules and resolves within 2 weeks (Goldberg *et al.* 2008). A long term follow up of rhamnolipid-induced inflammatory response may give more detailed information on the pulp response scenario. The tooth section model that was employed herein has its own limitation; only innate immune response can be investigated making it difficult to predict whether rhamnolipid can be implicated in adaptive immunity. Further study is also required to assess its ability to induce reparative dentine formation in the exposed pulp.

IL-1beta is a double-edged sword inflammatory cytokine, i.e. it is essential to resist infections but also exacerbates damage during chronic disease and acute tissue injury (Dinarello 2010).

Beside innate immune cells, IL-1beta can be produced by pulp fibroblasts and vascular endothelial cells (Barkhordar *et al.* 2002) but not odontoblasts (Yumoto *et al*. 2018). Research has shown a higher level of IL-1beta in inflamed pulp tissue than in healthy controls (Silva *et al.* 2009) and the fold increase was comparable to the current finding. Similarly, pulp fibroblasts derived from infected tooth were found to contain 2.5 fold greater amount of IL-1beta and showed the potential to synthesise around 80 % greater amounts of collagen compared to fibroblast cells of healthy pulp (Barkhordar *et al.* 2002).

Contradicting with the current finding that rhamnolipid did not show significant potential to induce higher level of TNF-alpha, Rha-Rha-C14-C14, which is structurally quite similar to the rhamnolipid exotoxin from *P. aeruginosa*, exhibited strong stimulatory activity on human mononuclear cells to produce TNF-alpha, a pleiotropic inflammatory cytokine (Andrä *et al.* 2006). Another study demonstrated that rhamnolipids induced copious release of IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor from epithelial cells at sub-cytotoxic levels (Bédard *et al.* 1993).

The anti-inflammatory cytokine; IL-10 was detected in all of the samples tested showing a trend toward controlling the immune response. However, its level in the rhamnolipid treated section was slightly ($p < 0.05$) lower than the untreated control.

The antimicrobial factor defensin-beta2 was suppressed in the presence of rhamnolipid including bacterially infected sections co-cultured with rhamnolipid. The expression and release of this antimicrobial peptide is crucial to neutralise pathogens (Chromek *et al.* 2006; Abdel-Mawgoud *et al.* 2010). It has been reported that rhamnolipids from *P. aeruginosa* supernatant are able to subvert a flagellin-induced human defensin-beta2 response in keratinocytes by interfering with host-cell calcium signalling, targeting protein kinase C (PKC) and therefore can manipulate host innate immune response (Dössel *et al.* 2012).

The role played by rhamnolipid in assessing wound healing and tissue regeneration (Stipcevic *et al.* 2006; Shen *et al.* 2016) encouraged looking at its effect on growth factor (TGF-beta). This signalling molecule plays a vital role in stem cell differentiation, chemotaxis and apoptosis in monocytes, epithelial, mesenchymal and neuronal cells (Gold *et al.* 2000; Kubiczkova *et al.* 2012; Niwa *et al.* 2018). TGF-beta induced myofibroblasts were selectively inhibited by rhamnolipid (Shen *et al.* 2016) suggesting rhamnolipid as a novel anti-scar

therapy, however the mechanism still needs to be clarified, which might involve TGF-beta-rhamnolipid relationship. Specifically in pulp fibroblasts, TGF-beta2 has been reported to induce the synthesis of collagen matrix (Chan *et al.* 2005). Rhamnolipid treated section demonstrated a reduced, though non-significant, reduction in the TGF-beta level in comparison to the untreated control or the infected sections with or without rhamnolipid.

5.5 Conclusion

This study successfully employed an existing *ex-vivo* tooth model to assess the biosurfactant antimicrobial activity in co-culture with bacterially infected tooth sections. Rhamnolipid at 0.097 mg/mL demonstrated powerful potential to prevent tooth section infection in co-culture with *S. anginosus* and *S. constellatus* in serum-free medium. The resulting inflammatory response pattern showed a trend of moderate inflammation balanced by an induction of immunosuppressant cytokine. It was also demonstrated that rhamnolipid solution could tolerate serum concentration ≤ 5 % in culture medium without affecting its activity. This means that rhamnolipid can be further investigated as a pulp therapy antimicrobial adjuvant.

Lp-BS was found to be potentially toxic eliciting cell apoptosis early (24 h) post incubation. Its effect on tissue was noxious particularly with prolonged incubation (48 h). *Lp*-BS fraction obtained from purification by FPLC can be assayed in the future for its toxicity and antiadhesive activity as endodontic agent.

6 Chapter 6 General summary and future direction

6.1 General summary

As an attempt to treat and preserve infected pulp, vital pulp therapy has been introduced and many agents have been in use. However, failure has been reported mainly due to microbial contamination. Developing new strategies to combat microbial infection is needed for a more successful outcomes. Hence the aim of this thesis was to investigate the possible use of probiotic bacteria in the control of dental infection; more specifically endodontic infection. In particular, the intention was to extract a biosurfactant compound from the potential probiotic, characterised and investigated it further as an antimicrobial/antiadhesive material that can be used in conjunction with vital pulp therapy. A commercially sourced biosurfactant, rhamnolipid was also included in this study to test its antimicrobial effect against endodontic pathogens.

Five probiotic strains were selected because they have shown promising results in combating bacterial infections (Falagas and Makris 2009). Of the probiotic strains assayed in this project, *L. plantarum* demonstrated the most significant growth inhibitory effects against SAG and *E. faecalis*. Other strains also revealed some inhibitory effects against one or more of the tested pathogens. However *L. plantarum* was selected for further study because it affected the growth of all four test strains. Moreover, interesting promising therapeutic benefits for *L. plantarum* have been documented in the literature for non- endodontic infection which encouraged investigation for its use in clinical dental applications. For instance, *L. plantarum* has been shown to benefit host immune function via stimulation of gut-associated and other lymphoid tissues, creation of an acidic environment in the gut which is hostile to many pathogens, enhancement of mucosal barrier function, induction of T-cell apoptosis in the lamina propria and inhibition of inflammatory cytokines (Naruszewicz *et al.* 2002; Fedorak and Madsen 2004; Pathmakanthan *et al.* 2004; Ko *et al.* 2007; Wullt *et al.* 2007; Connelly 2008). Moreover, *L. plantarum* has also been attributed with the potential to rapidly inhibit pathogens either by competing with pathogens for receptor space on epithelial tissue or depleting the space for pathogens to grow (Klarin *et al.* 2008). It was also reported to produce a bacteriocin which inhibits *Listeria monocytogenes* and to reduce *Clostridium difficile*-associated diarrhoea by stimulating butyrate and short chain fatty acid production (Olasupo

1998; Niku-Paavola *et al.* 1999; Hutt *et al.* 2006; Rokka *et al.* 2006). *L. plantarum* revealed, for the first time, the potential to inhibit endodontic isolates in this study, this effect was reported *in-vitro*. The *in-vivo* situation may differ particularly when biokinetics factors such as host serum effect are considered (Li *et al.* 2017). Moreover, the pathogen biofilm phenotype, which can be 1000 time more resistant is an additional important factor that should be taken into account when considering the *in-vivo* situation (Hall and Mah 2017).

It was hypothesised that the observed antimicrobial effect of *L. plantarum* against SAG and *E. faecalis* might be due to the production of biosurfactant. Therefore biosurfactant compounds were extracted at four different time points representing the different phases of *L. plantarum* growth. The sample collected at the mid-exponential phase (designated *Lp*-BS) demonstrated the maximum surface tension lowering effect and therefore it was decided to proceed with this time point for further biosurfactant production and extraction. Beside lowering surface tension, *Lp*-BS also revealed an emulsifying activity against a number of hydrocarbons (Gharaei-Fathabad 2011).

The extraction was performed according to a previously optimised protocol (Gudina *et al.* 2010) with minor modification. Among varieties of extraction protocols (Satpute *et al.* 2010; Varjani and Upasani 2017), this method was chosen because it did not involve the use of harsh chemicals like strong acid or alcoholic solvents (Das *et al.* 2014) which may affect the integrity and activity of the obtained product. However, this method of extraction was basic and the product obtained cannot be considered pure, even following dialysis to get rid of low molecular weight contaminants (> 1000 Da) including the extracting buffer (PBS). It is important to consider and appreciate that this project was developed from 'the ground up' and that working in biosurfactant research is a complex multidisciplinary area of effort. There is a significant requisite for analytical scheme development and production optimisation, which may be challenging and muddled by some research groups. This is something that has been highlighted and commented upon by other authors (Marchant *et al.* 2014; Elshikh *et al.* 2016). Partial characterisation by means of FT-IR supported by anthrone and protein assays revealed a glycoprotein nature of *Lp*-BS and this correlated with the results obtained by other researchers (Tahmourespour *et al*. 2011a; Savabi *et al.* 2014). However, FT-IR as a standalone method is limited for the purpose of characterisation (Barth 2007). Nuclear magnetic

resonance (NMR) spectroscopy alongside other techniques such as HPLC-MS could be applied to more fully characterise such macromolecules (Elshikh *et al.* 2016).

Proteomic analysis of *Lp*-BS revealed a rich and heterogeneous protein nature. Based on database comparisons, three moonlighting proteins were identified (Chapter 3) that had proposed roles for facilitating adhesin-like activity; namely elongation factor Tu, enolase 1, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The detection of such protein-binding proteins in the *Lp*-BS mixture may suggest that there is a role that can be played by this compound in binding to pulp ECM proteins and thus probably blocking bacterial attachment and colonisation sites. In an *in-vitro* antiadhesive assay using glass surface coated with the biosurfactant, *Lp*-BS exerted significant antiadhesive effect against SAG and *E. faecalis* when applied as a surface coating at 20 mg/mL. This finding might indicate a novel and promising non-pharmacological antibacterial activity that can be played by this compound which has not been reported for any of the current pulp therapy agents. It further highlights the possible involvement of the identified adhesin-like proteins in the reported antiadhesive property. At lower concentration of 10 mg/mL, SAG attachment was reduced though not significantly indicating a dose-dependent response similar to that demonstrated with some other biosurfactants (Cao *et al.* 2009; Jemil *et al.* 2017). Glass surface *Lp*-BS coating was successfully characterised for the first time by staining with fluorescent stain (SYPRO Ruby stain) and this demonstrated a successful coating.

Although a number of biosurfactants have been shown to possess antimicrobial activity (Santos *et al.* 2016), the *Lp*-BS investigated herein did not demonstrate antimicrobial effect against SAG or *E. faecalis* at a wide range of concentrations between 0.1-50 mg/mL. This lack of antimicrobial activity might be due to the strain-specificity or to the biosurfactant extraction method used. It might be that the complex protein-carbohydrate nature of the glycoprotein *Lp*-BS blocks the biosurfactant functioning site and hence further purification of *Lp*-BS may be necessary. In addition, *Lp*-BS was obtained during the mid-exponential phase and it may be that this extract is less efficient as antimicrobial than samples that would be obtained later in the growth curve where the exhaustion of nutrient may encourage the production of competing molecules with antimicrobial activity.

Lp-BS was extracted from a "generally regarded as safe" (GRAS) probiotic, and therefore it could be hypothesised to be safe when applied to the biological tissue. However, *Lp*-BS demonstrated significant toxicity against rat pulp cells even at low concentration of 5 mg/mL after 72 h incubation. Cell apoptosis was shown to be induced early within the first 24 h incubation with *Lp*-BS at 10 and 5 mg/mL. It is possible that because cells were grown as a monolayer, they may be more sensitive to the toxic effect of the treatment. Therefore, *Lp*-BS was applied to pulpal tissue utilising a previously developed and characterised *ex-vivo* tooth section model (Roberts *et al.* 2013). Co-culture of the tooth sections with *Lp*-BS 0.625-10 mg/mL for 24 h resulted in no significant difference ($p > 0.05$) in the cell counts and histological appearance between the sections treated with the different *Lp*-BS concentration and the untreated control sections. This finding would tend to suggest an apparent lack of toxicity of *Lp*-BS to the pulp tissue. However, low mRNA yield and down regulation of most of the analysed inflammatory markers genes obtained at 10 mg/mL treatment suggested a cell abnormality which might be due to early apoptosis via induction of DNA damage and cell death (Thomas *et al.* 2015). Indeed, when the effect of treatment was examined for 48 h at 10 mg/mL *Lp*-BS, significant toxicity was demonstrated, with obvious histological changes and low cell counts in comparison to the untreated control tooth sections. It is difficult to determine which specific component or components were responsible for the toxicity considering the complex composition and the crude nature of *Lp*-BS. It might be that certain *Lp*-BS components integrated with the cell membrane and this might have triggered molecular pathways that ultimately result in cellular toxicity (Zhang 2018). Alternatively, contaminant molecules of the crude *Lp*-BS may have led to cell-death. Because of the toxicity encountered with the use of *Lp*-BS, it was decided not to continue with this crude product. Further purification was therefore needed and was achieved partially by fractionation chromatography based on size which resulted in four elution pools. The first largest obtained fraction preserved the biosurfactant compound indicated by the emulsifying activity demonstrated by this fraction. It was supposed that biosurfactant could form micelle macromolecules hat can be eluted early leaving the smaller contaminants to be collected later (Mukherjee *et al.* 2009). The successfully partially purified fraction containing the biosurfactant is available for further testing particularly in the context of toxicity. However, this was not possible within the confines current study.

Commercially available rhamnolipid, was initially employed in this research as a biosurfactant positive control. This glycolipid has been extensively studied in different biomedical fields (Irfan-Maqsood and Seddiq-Shams 2014; Chong and Li 2017). However, its application within endodontic therapy has never been explored. Rhamnolipid revealed powerful antimicrobial activity against the SAG members studied with MICs of 0.097 mg/mL against *S. anginosus* and 0.048 mg/mL against *S. constellatus* and *S. intermedius*. *E. faecalis* was found difficult to be inhibited at the lower concentrations tested and required a high concentration of 50 mg/mL to be completely inhibited. Though rhamnolipid did not effectively inhibit *E. faecalis* growth at the lower concentration, its ability to inhibit the "pioneer" SAG strains is a significant finding in the context of the development of endodontic biotherapy and it may be that rhamnolipid could be used to reduce the levels of *E. faecalis* via an effect on the pioneer species. This would need to be investigated further. In addition to antimicrobial activity, rhamnolipid showed the potential to reduce *S. anginosus* and *S. intermedius* attachment to acrylic discs coated with rhamnolipid at 50, 0.097 and 0.048 mg/mL. These promising results justified the further testing of rhamnolipid on pulp cells and tissue. Rhamnolipid cytotoxicity on rat pulp cells was tested in serum containing medium. Further investigations were performed on rhamnolipid immunogenicity on the pulp tissue also in serum containing medium. A further application of rhamnolipid on bacteria-infected tooth section was performed to assess its *ex-vivo* antimicrobial activity, again in serum containing culture medium. But, an inactivation of rhamnolipid antimicrobial activity was encountered which was proposed to be due to serum effect (Jiang *et al.* 2014). By omitting serum from the culture medium and co-culture *S. anginosus* and *S. constellatus*-infected tooth sections with rhamnolipid at 0.097 mg/mL, significant antimicrobial activity was observed. *S. anginosus* and *S. constellatus* were chosen for the tooth section co-culture experiments because they were shown to be capable of surviving in the modified culture medium (DMEM + 10 % BHI).

The *ex-vivo* tooth model was also used to investigate the immune response of the dental pulp cells to rhamnolipid treatment. Of the four pro-inflammatory cytokines (IL-6, IL-1beta, TNF-alpha and IL-18) tested using an ELISA method within 24 h-culture supernatants, only IL-1beta demonstrated higher levels (> 60 pg/mL) of the released cytokine in rhamnolipid-treated infected or non-infected tooth sections. There was no significant difference in the amount of the anti-inflammatory IL-10 when rhamnolipid-treated sections and the bacteria-infected

control sections were compared. These findings might suggest a mild controlled inflammatory pulp response to rhamnolipid treatment. Clinically, mild inflammatory response together with limited bacterial contamination is a requirement for pulp tissue repair (Goldberg *et al.* 2008), these two properties can be provided by rhamnolipid treatment. Furthermore cell differentiation in response to rhamnolipid treatment can be investigated. The *ex-vivo* tooth model employed in this study provides limited capacity to explore the immune response due to the possible loss of some or all of the immune cells, no active blood supply and inability to follow the chronic immune response.

Rhamnolipid treatment did not enhance the release of the growth factor TGF-beta or the antimicrobial peptide defensin-beta2. This finding is contrary to previous reports on the involvement of rhamnolipid in tissue regeneration and wound healing (Stipcevic *et al.* 2006). Regarding defensin-beta2, it has been reported that rhamnolipid released from *P. aeruginosa* was considered as a virulent factor and was demonstrated to suppress the release of the antimicrobial molecule (Dössel *et al.* 2012). However further studies are required to analyse the effect of rhamnolipid on this molecule at different time points (earlier to or later than 24 h) and at the gene expression level.

Although rhamnolipid was applied to the tooth section in serum-free medium, residual tooth section serum did not seem to inactivate rhamnolipid demonstrated by the prominent antimicrobial activity in the co-culture *ex-vivo* experiment. Rhamnolipid was shown to retain activity at FBS concentration up to 5 % in the culture medium. Higher concentrations of 10-20 % FBS appeared to result in rhamnolipid inactivation as demonstrated by a dramatic attenuation of its cytotoxic effect. Serum protein binding is a crucial dilemma in the context of the delivery and bioavailability of different medicaments (Vuignier *et al.* 2010). It can reduce the bioavailability of the drug to the target tissue but, at the same time, it can work as a reservoir allowing a controlled-release drug formulation.

Overall, a novel application of rhamnolipid as a potential endodontic antimicrobial agent was highlighted by the work reported in this thesis that showed promising results in the prevention of oral microbial infection particularly in case of Streptococcal pulpal infection. The further development of this material for optimum delivery may result in a new pulp therapy which could be effective in reducing the cases of failed pulp therapy due to bacterial contamination and therefore reducing the need for additionally extensive and invasive root

canal therapy, which results in irreversible loss of pulpal vitality and is associated with reduced tooth longevity. In addition, non-antimicrobial pharmacotherapy targeted at specific virulence factors (such as antiadhesion) may play a preventative or therapeutic role in the management of endodontic infections. Therefore, future directions of research could focus on *Lp*-BS adhesion proteins and their possible involvement as an effective strategy to reduce microbial adhesion and combating colonisation by pathogenic microorganisms, not only in the pharmaceutical and biomedical field but also in other areas, such as food industry and cosmetics. Although probiotic *Lactobacillus* therapy has been widely investigated, their effects on SAG and *E. faecalis* is still not satisfactory and the result obtained in this thesis with *L. plantarum* is encouraging for ongoing further investigations.

6.2 Future directions

1- To further investigate the role of *L. plantarum* and its products in the management of endodontic infections, work should be undertaken that more fully reflects the *in-vivo* situation. These may include assays for its adhesion potential to the dental/pulp tissues and studies of how the oral clearance mechanisms may be resisted. Moreover, the antimicrobial activity of *L. plantarum* should be tested against pathogens attached to the host tissue; where their pathogenicity and virulence can be more prominent (Bauer and Shafer 2015).

2- The applicability of *L. plantarum* biosurfactant as tensioactive bioemulsifier compound could be further investigated in the field of pharmaceutical technology and formulation and in the context of bioremediation.

3- FPLC biosurfactant-containing fraction can be further studied in the future for its antimicrobial, antiadhesive and cytotoxic effects. It can be assayed for the presence of adhesion proteins by mean of Mass spectrometry. The target adhesin-like protein can be antibody-blocked and the antiadhesive activity can be compared. The obtained fraction also may offer less cytotoxicity due to the removal of the contaminants.

4- The reported *Lp*-BS apoptotic effect could prove promising in controlling cancerous cells as has been reported for other similar biosurfactants (Zhao *et al.* 2000; Kim *et al.* 2007). Therefore, further studies can be conducted considering the effect of the obtained biosurfactant on cancerous and healthy cell lines.

5- A wider range of bacterial strains that involve in endodontic infection such as *Prevotella intermedia*, *Fusobacterium nucleatum*, *S. mitis*, *S. sanguinis* and others need also to be studied for their pattern of tooth infectivity and response to rhamnolipid treatment employing the *ex-vivo* model. Because of the heterogeneity of endodontic microbiology (Singh 2016) and difficulty to cover all possible infective strains, clinical samples obtained from primary pulpitis cases can be tested instead of testing individual strain separately.

6- *In-vivo* application of rhamnolipid can be conducted on experimental animals to investigate its immune-modulatory effect. It can also be applied on individual immune cell line such as pulp extracted macrophages or fibroblasts and followed for gene

expression and release of inflammatory cytokines. Its effect on pulp stem cells differentiation should also be studied.

7- The effect of serum concentration on rhamnolipid antimicrobial activity requests to be tested in the future. This can be achieved by culturing the bacterial isolates in rhamnolipid containing culture medium with different serum percentages and looking at bacterial growth curves in comparison to the control.

8- The property of rhamnolipid binding to serum protein can be explored further which might provide a way of delivering rhamnolipid in a controlled slow release level especially its *in-vivo* administration into experimental animals has gained safety. Rhamnolipid has been also declared in a patent published in 1988 (Ishigami *et al.* 1988) to be successfully formulated as liposome. The author suggested a wide range of applications for such formulation including drug-delivery. Taking the antimicrobial/antiadhesive and other immune effects of rhamnolipid obtained in this thesis into account, formulating rhamnolipid into liposome might be a promising way of delivering rhamnolipid. This might offer reducing rhamnolipid cytotoxicity, enhancing its antimicrobial property and achieving more stability (Sercombe *et al.* 2015).

9- Rhamnolipid could be incorporated into hydrogel for ease of application into the pulp or the root canal. This formulation can function as a mechanical matrix, a requirement for pulp capping material, supporting pulp cells adherence and multiplication therefore allows for normal pulp development (Klein-Júnior *et al.* 2018). The potential probiotic can also be laden into a gel carrier. The loaded gel can further be applied to treat periodontitis (Stipcevic *et al.* 2000) where it may be placed in periodontal "pockets", or voids between the tooth and the gingiva where periodontitis occurs (Vyas *et al.* 2000) and so the physical and antimicrobial properties of the gel could be assessed for this application with the potential to optimise the material properties for this oral location.

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Appendix I: Mass spectrometric analysis data sheet of the *Lp*-BS 10 proteins bands analysed

Note: accession number obtained by search using Uniprot. Score, the sum of the ion scores of all peptides that were identified; Coverage, the percentage of the protein sequence covered by identified peptides; # Unique Peptides, the number of peptide sequences that are unique to a protein group; # Peptides, the total number of distinct peptide sequences identified in the protein group; # PSM's, the number of peptide spectrum matches; MW, molecular weight.

Band # 1

Accession	Description	Score	Coverage	# Unique Peptides	# Peptides	# PSMs	MW [kDa]
Q88XY8	Elongation factor G	369.24	60.89	36	36	150	77.0
F9UMJ6	ATP-dependent Clp protease	224.88	50.66	39	40	82	92.6
Q88U67	Probable phosphoketolase 1	175.75	35.03	23	24	68	88.7
Q88YM5	60 kDa chaperonin	161.72	47.69	23	23	50	57.4
F9UPA5	Transpeptidase-transglycosylase (Penicillin binding protein 1A)	155.01	39.77	30	30	59	83.1
Q88YL7	Protein translocase subunit SecA	149.68	35.32	25	25	56	89.5
F9UPM3	Pyruvate kinase	129.83	41.98	20	20	45	62.8
Q88VM0	Chaperone protein DnaK	119.02	42.12	24	24	39	66.7
F9UPH5	DNA topoisomerase 4 subunit A	104.44	27.82	24	24	41	91.6
F9UMB1	DNA helicase	102.79	33.07	23	23	40	87.9
Q88XZ2	DNA-directed RNA polymerase subunit beta'	95.63	28.85	32	32	43	135.2
F9ULD9	ATP-dependent Clp protease, ATP-binding subunit ClpL	91.80	31.11	17	17	27	77.7
Q88VK7	Translation initiation factor IF-2	90.84	29.60	22	22	36	93.6
F9UMU5	ATP-dependent DNA helicase	85.38	33.91	24	24	33	85.0
F9UU18	DNA helicase	83.83	27.31	19	19	30	87.8
F9US38	DNA gyrase subunit A	74.47	18.76	16	16	28	94.5
Q88V10	Alanine--tRNA ligase	73.48	25.00	19	19	26	97.2
Q88V16	Endonuclease MutS2	71.34	22.36	15	15	24	87.2
F9UN58	ATP-dependent Clp protease, ATP-binding subunit ClpE	69.37	28.55	19	19	26	81.7
F9UQ86	Translation elongation factor, GTPase,TypA/BipA family	68.95	25.33	12	12	24	68.4
Q88VS3	Glycine--tRNA ligase beta subunit	66.68	29.11	20	20	29	78.5
Q88S87	Probable phosphoketolase 2	64.09	25.88	21	22	33	89.6
F9UPV0	GTP pyrophosphokinase / pyrophosphohydrolase	63.47	25.70	21	21	29	86.2
Q88YH3	Enolase 1	62.86	24.21	9	9	20	48.0
Q88XA9	Leucine--tRNA ligase	60.56	21.91	19	19	26	92.6
Q88YH5	Phosphoglycerate kinase	60.41	37.25	14	14	26	42.8
F9UMD4	Aminopeptidase	60.31	20.62	16	16	24	93.9
Q88VE1	Trigger factor	59.81	32.50	13	13	23	49.4
F9UP85	Maltose phosphorylase	57.83	20.45	14	14	20	85.6
Q88VJ5	Elongation factor Ts	57.23	40.07	10	10	17	31.6
F9UN60	Phosphoenolpyruvate-protein phosphotransferase	52.30	30.03	13	13	19	63.1
Q88YC0	Xaa-Pro dipeptidyl-peptidase	52.03	24.60	17	17	20	91.5

Q88VJ2	D-lactate dehydrogenase	51.06	44.88	14	14	23	37.2
F9UPL0	30S ribosomal protein S1	49.88	34.03	15	15	20	47.1
Q88UX7	Valine--tRNA ligase	49.67	20.92	19	19	25	101.5
F9URD9	Cell wall hydrolase/muramidase	47.92	19.75	11	11	16	82.1
F9ULZ0	Phosphoglucomutase	45.48	33.91	17	17	20	63.5
F9UM10	Glyceraldehyde 3-phosphate dehydrogenase	43.26	28.24	9	9	15	36.4
Q88VX7	Chaperone protein ClpB	42.55	13.73	12	13	16	96.5
Q88VE0	Elongation factor Tu	42.31	33.67	11	11	17	43.4
F9UPI3	DNA topoisomerase 1	41.22	21.12	16	16	20	80.7
F9UP05	Signal recognition particle receptor FtsY	40.28	23.44	12	12	18	53.6
Q88VJ4	30S ribosomal protein S2	39.86	37.83	10	10	14	30.2
F9UTT2	Fructose-bisphosphate aldolase	39.72	22.30	5	5	11	30.9
F9ULT0	Ribonucleoside-diphosphate reductase	38.38	14.29	11	11	15	82.1
F9UNP5	DNA polymerase I	36.95	11.79	9	9	13	98.7
P77886	Carbamoyl-phosphate synthase pyrimidine-specific large chain [CARB_LACPL]	35.95	13.42	13	13	17	115.7
Q88XZ3	DNA-directed RNA polymerase subunit beta	35.80	14.49	16	16	16	134.4
Q88WU9	Threonine--tRNA ligase	33.92	20.64	14	14	17	73.8
Q88V89	Isoleucine--tRNA ligase	33.07	15.56	13	13	16	106.3
Q88Z31	ATP-dependent zinc metalloprotease FtsH	32.34	16.38	12	12	15	80.8
Q88WR2	Phenylalanine--tRNA ligase beta subunit	32.03	19.50	14	14	21	88.1
Q88UW5	Glutathione biosynthesis bifunctional protein GshAB	31.60	17.71	13	13	14	83.0
F9UTA3	GMP reductase	31.22	29.24	9	9	13	39.9
Q88YE7	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	28.23	15.70	9	9	12	65.4
Q88Z76	CTP synthase	27.64	14.71	8	8	12	59.7
Q88Z97	Methionine--tRNA ligase	25.58	19.03	13	13	14	76.7
F9UM19	Ribonuclease R	25.31	13.32	11	11	15	92.3
F9URU9	Extracellular transglycosylase, with LysM peptidoglycan binding domain	24.58	17.51	6	6	10	35.0
P56512	L-lactate dehydrogenase 1	24.26	25.62	8	8	10	34.2
Q88UI4	Glucose-6-phosphate isomerase	23.74	21.56	8	8	9	49.8
Q88YH4	Triosephosphate isomerase	23.54	28.17	6	6	9	27.0
Q88YW9	50S ribosomal protein L1	23.48	24.89	6	6	8	24.8
F9UM05	Oligopeptide ABC transporter, substrate binding protein	23.36	13.45	7	7	10	61.0
F9UPD2	Carbamoyl-phosphate synthase (glutamine-hydrolyzing)	22.87	12.19	10	10	10	94.3
Q88UU3	ATP synthase subunit beta	22.39	12.63	5	5	8	50.8
Q88VK1	Proline--tRNA ligase	22.09	15.29	9	9	10	63.5
Q88XY9	30S ribosomal protein S7	21.83	26.28	3	3	7	17.8
F9UL38	Cation transporting P-type ATPase	21.64	12.56	9	9	9	95.3
F9UQZ0	Prophage P2a protein 6 endonuclease	21.55	24.36	9	9	10	40.6
Q88W32	Probable manganese-dependent inorganic pyrophosphatase	21.45	29.13	8	8	8	33.6
F9US37	DNA gyrase subunit B	21.44	13.43	9	9	10	72.1
Q88VQ8	Aspartate--tRNA ligase	21.11	15.72	10	10	13	67.6
F9UUA0	Extracellular protein,gamma-D-glutamate-meso-diaminopimelate muropeptidase	19.49	13.24	4	4	7	36.8
F9UNY9	Primosomal protein N'	19.48	11.43	10	10	11	90.0

F9UMH1	Asparagine synthase (Glutamine-hydrolysing)	19.47	14.53	9	9	12	73.1
Q88T35	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2	19.22	30.87	5	5	7	26.1
Q88WA8	Asparagine--tRNA ligase 2	18.84	10.88	4	4	7	49.8
Q88YD2	Polyphosphate kinase	18.68	10.17	8	8	10	82.2
F9UP39	3-oxoacyl-[acyl-carrier-protein] synthase 2	18.64	11.95	4	4	6	42.4
F9UNI1	GTP cyclohydrolase-2	18.53	12.38	4	4	6	43.6
F9UL45	PTS system, mannose-specific EIIAB component	17.69	12.96	4	4	7	35.3
Q88XW0	DNA-directed RNA polymerase subunit alpha	17.62	14.97	5	5	7	34.8
F9ULY4	UTP--glucose-1-phosphate uridylyltransferase	16.70	21.24	6	6	6	34.2
Q88XX4	50S ribosomal protein L5	16.58	26.11	5	5	9	20.2
Q88XY3	50S ribosomal protein L2	16.51	39.78	10	10	16	30.2
F9UPK7	DNA-binding protein	16.39	40.66	3	3	5	9.6
F9US12	Cell surface protein, LPXTG-motif cell wall anchor	16.01	5.04	3	3	5	89.5
Q88XY6	50S ribosomal protein L3	15.77	17.22	3	3	7	22.7
F9UNZ9	Phosphatase, dihydroxyacetone kinase family	15.74	8.80	5	5	6	60.7
Q88YW7	50S ribosomal protein L7/L12	15.69	52.46	6	6	7	12.6
Q88Y74	GMP synthase [glutamine-hydrolyzing]	15.63	14.67	8	8	9	57.4
F9UQH9	Extracellular protein, DUF336 family	15.57	28.82	4	4	5	18.0
Q88UU1	ATP synthase subunit alpha	15.38	9.33	4	4	5	54.5
F9UP45	Enoyl-[acyl-carrier-protein] reductase [NADH]	14.15	23.41	6	6	7	26.9
F9UMJ0	Acetolactate synthase	14.03	9.29	4	4	6	61.2
F9US18	Anaerobic ribonucleoside-triphosphate reductase	13.83	8.24	6	6	7	83.7
F9UT53	Cystathionine beta-lyase / cystathionine gamma-lyase	13.70	13.91	5	5	6	40.8
Q88X53	Arginine--tRNA ligase	13.44	11.57	6	6	7	62.9
Q88XP7	Glutamyl-tRNA(Gln) amidotransferase subunit A	13.19	12.53	6	6	6	52.1
Q88XW9	30S ribosomal protein S5	13.06	30.72	6	6	6	17.3
Q88UV8	D-alanine--D-alanine ligase	12.85	12.70	4	4	5	41.3
F9URA6	Formate C-acetyltransferase	12.41	7.86	7	7	7	91.6
Q88W26	ATP-dependent protease ATPase subunit HslU	12.40	5.93	2	2	4	52.6
Q88XY0	30S ribosomal protein S3	12.18	25.81	5	5	6	24.2
Q88XZ7	Serine--tRNA ligase 2	12.15	11.29	4	4	4	48.1
Q88WN5	50S ribosomal protein L21	11.84	27.45	2	2	4	11.0
Q88VM1	Chaperone protein DnaJ	11.69	15.26	4	4	4	40.5
Q88XP6	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	11.31	9.70	5	5	5	53.2
F9UR64	NADH peroxidase	11.16	8.43	4	4	5	48.2
F9UNV9	Glutamine synthetase	11.13	12.28	5	5	5	50.9
F9UUC0	Endopeptidase PepO	10.81	6.90	5	5	6	71.9
F9UP42	Acetyl-CoA carboxylase, biotin carboxylase subunit	10.74	10.61	4	4	4	50.7
F9UNV2	Bifunctional protein: transcription regulator sugar kinase, ROK family	10.58	13.13	3	3	3	33.7
Q88WG0	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta 2	10.37	10.32	2	2	3	30.9
Q88VE2	ATP-dependent Clp protease ATP-binding subunit ClpX	10.33	12.83	5	5	5	46.5
F9UQC3	Cell division protein FtsZ	10.31	8.90	4	4	4	45.0
F9ULV5	ABC transporter, ATP-binding protein	10.17	5.76	3	3	3	74.1

F9UMC8	Alkaline shock protein	10.09	23.57	2	2	3	15.8
Q88XY5	50S ribosomal protein L4	10.06	15.94	3	3	4	22.6
F9US03	Excinuclease ABC, subunit A	9.99	5.51	4	4	4	89.5
F9UR85	Aspartate-semialdehyde dehydrogenase	9.98	7.37	2	2	3	38.2
F9UQA0	Extracellular protein, NlpC/P60 family,gamma-D-glutamate-meso-diaminopimelate muropeptidase	9.88	5.65	2	2	3	48.3
Q88XF3	Peptide chain release factor 3	9.81	8.76	4	4	4	59.5
F9UNG7	Hypothetical membrane protein	9.66	3.64	3	3	4	91.9
F9UNS5	6-phosphogluconate dehydrogenase, decarboxylating [F9UNS5_LACPL]	9.62	5.02	2	2	3	52.9
F9UN64	Serine-type D-Ala-D-Ala carboxypeptidase	9.54	8.25	3	3	3	45.2
F9UMX4	UDP-galactopyranose mutase	9.40	13.94	5	5	6	43.2
F9UL33	N-acetylglucosamine-6-phosphate deacetylase	9.34	10.85	4	4	5	41.4
F9UL89	Prophage P1 protein 2, mitogenic factor, cell surface lipoprotein	9.20	11.37	4	4	4	41.8
F9UT87	Cyclopropane-fatty-acyl-phospholipid synthase	9.00	8.56	3	3	3	45.3
F9UNU7	Transpeptidase (Penicillin binding protein 2B)	8.89	4.13	2	2	3	72.6
F9USY7	Maltodextrin-binding protein	8.83	6.71	3	3	3	45.6
F9UQ67	Ribonuclease J	8.77	6.10	3	3	3	63.9
F9URG7	Glucose-6-phosphate 1-dehydrogenase	8.56	8.70	4	4	4	56.8
Q88VQ7	Histidine--tRNA ligase	8.34	11.97	5	5	5	47.9
F9UM84	Glutamine ABC transporter, ATP-binding protein	8.28	10.16	2	2	3	27.4
F9UN93	Xaa-His dipeptidase	8.06	7.49	3	3	3	50.8
F9UTG0	Glutathione reductase	7.88	4.95	2	2	3	48.2
Q88YE8	Phosphoglucosamine mutase	7.80	6.87	3	3	4	48.3
Q6LWD9	Nickase	7.79	4.08	3	3	3	80.0
Q88XB8	S-adenosylmethionine synthase	7.68	8.61	3	3	3	42.7
F9UL68	Aminopeptidase C	7.63	9.71	5	5	5	50.2
F9UQ80	Pyruvate carboxylase	7.61	2.36	2	2	3	127.1
F9UTJ5	Formate C-acetyltransferase	7.43	7.71	6	6	6	84.4
Q88XX1	50S ribosomal protein L6	7.42	16.85	3	3	3	19.3
Q88UT5	Serine hydroxymethyltransferase	7.30	7.04	3	3	3	44.4
F9UL91	Prophage P1 protein 5, superinfection exclusion (Cell surface N-anchored)	7.30	12.93	6	6	6	37.9
Q88XV9	50S ribosomal protein L17	6.60	20.47	2	2	2	14.2
Q88TV5	Tyrosine--tRNA ligase	6.20	5.74	2	2	2	47.0
F9UMV3	Transcription regulator, TetR family	5.98	14.66	3	3	3	21.6
F9UT66	CDP-glycerol glycerophosphotransferase / glycosyltransferase	5.93	3.94	4	4	4	108.2
F9UT98	Serine-type D-Ala-D-Ala carboxypeptidase	5.90	7.88	3	3	3	46.9
Q88VM6	D-alanine--D-alanyl carrier protein ligase	5.79	5.31	3	3	4	56.0
Q88V23	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase	5.66	14.41	3	3	3	24.5
Q88VJ6	Uridylate kinase	5.51	9.58	2	2	2	25.9
F9URQ9	Uncharacterized protein	5.41	4.04	3	3	4	76.2
F9ULW6	Ribosome hibernation promoting factor	5.40	11.17	2	2	2	21.7
F9USZ3	Maltose phosphorylase	5.37	2.51	2	2	3	86.5
Q88UU0	ATP synthase subunit delta	5.32	12.15	2	2	2	20.0
Q06115	Choloylglycine hydrolase	5.31	6.79	2	2	2	37.0

F9UTR7	NADH dehydrogenase	5.31	4.00	2	2	3	43.8
F9UPM8	Peptidase T	5.30	5.34	2	2	2	45.1
Q88X05	Foldase protein PrsA 1	5.10	7.05	2	2	2	32.6
F9UKZ6	DEAD-box ATP-dependent RNA helicase CshA	5.07	8.14	4	4	4	59.0
Q88V33	Probable transcriptional regulatory protein lp_2253	5.05	11.98	3	3	3	26.4
F9URK9	Protein-tyrosine phosphatase	5.04	8.99	2	2	2	30.0
F9URZ3	Hypothetical membrane protein	4.94	3.98	4	4	4	115.2
Q88UZ4	Protein RecA	4.92	6.84	2	2	2	40.6
Q88V80	UDP-N-acetylmuramoylalanine--D-glutamate ligase	4.87	4.79	2	2	2	50.1
Q88YI7	UvrABC system protein A	4.80	2.10	2	2	2	104.9
F9UM27	Phosphate acetyltransferase	4.76	7.38	2	2	2	34.4
F9UQH6	Catabolite control protein A	4.71	9.23	3	3	3	36.3
Q88RX6	tRNA uridine 5-carboxymethylaminomethyl modification enzyme MnmG	4.65	4.87	3	3	3	70.7
F9UQC4	Cell division protein FtsA	4.54	5.80	2	2	2	48.3
F9UQS9	Mannose-6-phosphate isomerase	4.49	5.92	2	2	2	35.9
F9URK6	NADPH-dependent FMN reductase family protein	4.48	8.82	3	3	3	47.4
Q88XX7	30S ribosomal protein S17	4.46	20.22	2	2	2	10.3
F9UQH2	Ribokinase	4.42	6.54	2	2	2	31.7
F9UNL2	ABC transporter component, iron-sulfur cluster assembly protein SufB, iron regulated	4.27	4.06	2	2	2	52.6
Q88Z28	Lysine--tRNA ligase	4.24	5.21	3	3	3	57.6
Q88YY0	Glutamate--tRNA ligase	4.24	4.44	2	2	2	56.9
F9UTA5	Ribosome-binding ATPase YchF	4.21	5.74	2	2	3	39.9
F9UP94	Aminotransferase	4.19	4.99	2	2	2	43.8
Q88YX0	50S ribosomal protein L11	4.08	16.31	2	2	2	14.8
F9ULD3	Catalase	3.91	5.58	3	3	4	55.3
Q88XX2	30S ribosomal protein S8	3.90	14.50	2	2	2	14.6
F9US66	Serine protease HtrA	3.76	4.52	2	2	2	43.1
Q9RE01	Uracil phosphoribosyltransferase	3.72	7.66	2	2	2	23.0
F9UN23	Mannose-specific adhesin, LPXTG-motif cell wall anchor	3.41	2.18	2	2	2	107.9
F9UTV3	Multicopper oxidase	3.35	4.19	2	2	2	56.8
Q88VL9	Protein GrpE	3.25	11.56	2	2	2	21.4
F9UL42	Homoserine dehydrogenase	2.83	6.78	3	3	3	46.6
P77885	Carbamoyl-phosphate synthase pyrimidine-specific small chain	2.78	6.32	2	2	2	40.0
F9UQB9	Cell division initiation protein DivIVA	2.74	8.19	2	2	2	26.2
F9UP07	Signal recognition particle protein	2.70	3.72	2	2	2	53.9
F9UPS7	RNA polymerase sigma factor SigA	2.60	5.43	2	2	2	41.4
F9UQQ9	Cell shape determining protein MreB	2.03	4.80	2	2	2	35.8
F9UMS0	Citrate lyase alpha chain	1.96	6.25	3	3	3	54.9
Q88Z84	Ribose-phosphate pyrophosphokinase 1	1.91	4.29	2	2	2	35.9
Q88XU8	50S ribosomal protein L13	1.69	10.20	2	2	2	16.2
F9UPH6	DNA topoisomerase 4 subunit B	0.00	2.84	2	2	2	74.5

Band # 2

Accession	Description	Score	Coverage	# Unique Peptides	# Peptides	# PSMs	MW [kDa]
F9UPM3	Pyruvate kinase	324.72	86.52	40	40	114	62.8

Q88YM5	60 kDa chaperonin	254.78	79.11	38	38	80	57.4
Q88YE7	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	231.43	77.69	32	32	64	65.4
F9ULZ0	Phosphoglucomutase	216.91	73.74	35	35	62	63.5
Q88VE1	Trigger factor	158.23	60.91	26	26	51	49.4
F9UMH1	Asparagine synthase (Glutamine-hydrolysing)	124.64	54.50	23	28	42	73.1
Q88X53	Arginine--tRNA ligase	119.05	56.05	25	25	38	62.9
F9UQE9	Oligoendopeptidase F	109.03	50.25	22	22	32	67.7
Q88VE0	Elongation factor Tu	104.67	64.56	20	20	35	43.4
Q88Z76	CTP synthase	96.84	48.23	21	21	31	59.7
Q88YH3	Enolase 1	90.32	44.12	13	13	26	48.0
Q88VK1	Proline--tRNA ligase	89.35	43.94	19	19	30	63.5
F9UN60	Phosphoenolpyruvate-protein phosphotransferase	88.81	44.62	20	20	31	63.1
Q88WU9	Threonine--tRNA ligase	85.56	45.41	23	23	33	73.8
Q88VM0	Chaperone protein DnaK	81.62	48.39	20	20	26	66.7
F9UPL0	30S ribosomal protein S1	81.19	47.32	18	18	27	47.1
F9USW0	Linoleic acid isomerase	79.39	42.02	18	18	27	64.2
F9UM10	Glyceraldehyde 3-phosphate dehydrogenase	73.79	56.76	16	16	22	36.4
F9ULD9	ATP-dependent Clp protease, ATP-binding subunit ClpL	71.60	32.10	15	15	20	77.7
F9UQ67	Ribonuclease J	66.29	39.90	19	19	24	63.9
Q88YH5	Phosphoglycerate kinase	57.40	51.75	18	18	22	42.8
Q88Y74	GMP synthase [glutamine-hydrolyzing]	57.19	36.10	15	15	22	57.4
Q88XF3	Peptide chain release factor 3	56.66	32.00	12	12	17	59.5
F9UTV3	Multicopper oxidase	53.81	42.32	15	15	19	56.8
F9UPE1	Fibrinogen-binding family protein	53.09	35.74	15	15	18	64.4
F9UNZ9	Phosphatase, dihydroxyacetone kinase family	52.44	25.35	10	10	14	60.7
Q88VJ4	30S ribosomal protein S2	50.29	46.82	11	11	16	30.2
Q88XY8	Elongation factor G	48.99	28.37	15	15	19	77.0
Q88VJ2	D-lactate dehydrogenase	45.66	46.99	15	15	17	37.2
Q88UX2	Septation ring formation regulator EzrA	44.72	24.08	11	11	14	64.5
F9UM65	Pyruvate oxidase	44.44	25.89	13	13	16	63.6
F9UUC0	Endopeptidase PepO	41.66	27.43	14	14	15	71.9
F9ULT4	DNA polymerase III subunit gamma/tau	39.28	23.01	8	8	12	62.7
Q88Z28	Lysine--tRNA ligase	37.98	31.06	15	15	19	57.6
F9UTT2	Fructose-bisphosphate aldolase	37.48	45.64	10	10	13	30.9
F9UM05	Oligopeptide ABC transporter, substrate binding protein	35.09	23.82	11	12	13	61.0
Q88Z97	Methionine--tRNA ligase	35.02	21.52	12	12	13	76.7
F9UNY0	DNA repair protein RecN	33.94	22.52	11	11	11	62.8
F9USK0	Asparagine synthase (Glutamine-hydrolysing)	29.30	13.27	3	8	11	73.1
F9UNS5	6-phosphogluconate dehydrogenase, decarboxylating	28.76	19.46	8	8	9	52.9
Q88YY0	Glutamate--tRNA ligase	27.33	26.61	12	12	12	56.9
Q88XZ3	DNA-directed RNA polymerase subunit beta	25.93	7.49	8	8	10	134.4
F9URB5	1-deoxy-D-xylulose-5-phosphate synthase	24.24	16.12	7	7	9	63.7
Q88UI4	Glucose-6-phosphate isomerase	23.74	23.11	8	8	9	49.8
Q88UU3	ATP synthase subunit beta	23.42	19.49	7	7	7	50.8
Q88VQ8	Aspartate--tRNA ligase	23.17	22.91	13	13	14	67.6
F9URG7	Glucose-6-phosphate 1-dehydrogenase	22.99	20.85	8	8	9	56.8
Q88UU1	ATP synthase subunit alpha	22.13	17.86	8	8	9	54.5

Q88WA8	Asparagine--tRNA ligase 2	20.91	15.74	6	6	7	49.8
Q88RX6	tRNA uridine 5-carboxymethylaminomethyl modification enzyme MnmG	20.37	13.99	9	9	9	70.7
F9UN93	Xaa-His dipeptidase	19.84	13.49	5	5	7	50.8
F9UU91	Cell surface protein, CscB family	19.39	6.34	5	5	5	118.7
Q88W32	Probable manganese-dependent inorganic pyrophosphatase	18.38	26.21	7	7	7	33.6
F9UMD6	Type I restriction-modification system,methylation subunit	18.33	10.04	4	4	5	59.9
Q88YH4	Triosephosphate isomerase	17.12	25.40	5	5	5	27.0
P37063	Pyruvate oxidase	16.88	10.61	5	5	5	66.1
P56512	L-lactate dehydrogenase 1	16.84	31.87	9	9	9	34.2
Q88U67	Probable phosphoketolase 1	16.65	9.39	6	6	6	88.7
F9ULY4	UTP--glucose-1-phosphate uridylyltransferase	16.50	23.20	5	5	6	34.2
Q88XX4	50S ribosomal protein L5	16.37	48.89	7	7	8	20.2
Q88XY3	50S ribosomal protein L2	16.34	44.09	9	9	10	30.2
Q88VJ5	Elongation factor Ts	15.98	19.18	5	5	5	31.6
F9UP39	3-oxoacyl-[acyl-carrier-protein] synthase 2	15.91	24.63	6	6	6	42.4
Q88XY6	50S ribosomal protein L3	15.19	29.67	5	5	5	22.7
F9UQ86	Translation elongation factor, GTPase,TypA/BipA family	15.13	15.36	7	7	7	68.4
F9UP45	Enoyl-[acyl-carrier-protein] reductase [NADH]	14.26	30.95	6	6	6	26.9
F9UKZ6	DEAD-box ATP-dependent RNA helicase CshA	13.98	14.58	6	6	6	59.0
Q88VE9	UvrABC system protein C	13.91	9.11	5	5	5	68.7
Q88XY0	30S ribosomal protein S3	13.83	31.34	6	6	6	24.2
F9UMD4	Aminopeptidase	13.58	7.23	5	5	5	93.9
Q88YW9	50S ribosomal protein L1	13.50	22.71	5	5	5	24.8
Q88T35	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2	12.21	21.30	3	3	4	26.1
F9UN51	Oligopeptide ABC transporter, substrate binding protein	12.09	14.44	5	6	6	60.3
F9UNL2	ABC transporter component, iron-sulfur cluster assembly protein SufB, iron regulated	11.99	11.75	5	5	5	52.6
F9UQ91	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	11.81	16.01	6	6	6	45.9
F9UUA0	Extracellular protein,gamma-D-glutamate-meso-diaminopimelate muropeptidase	11.38	11.08	3	3	3	36.8
F9UTR7	NADH dehydrogenase	11.26	22.00	6	6	6	43.8
F9ULT0	Ribonucleoside-diphosphate reductase	11.20	5.55	3	3	3	82.1
F9URC8	Pyruvate oxidase	10.82	9.57	5	5	6	64.2
F9UT87	Cyclopropane-fatty-acyl-phospholipid synthase	10.43	10.83	3	3	3	45.3
Q88TV5	Tyrosine--tRNA ligase	10.36	10.53	4	4	4	47.0
F9UL45	PTS system, mannose-specific EIIAB component	10.04	12.04	4	4	4	35.3
F9ULE1	Pyruvate oxidase	9.98	7.53	3	3	3	63.8
Q88YE8	Phosphoglucosamine mutase	9.61	9.53	3	3	3	48.3
F9UNV9	Glutamine synthetase	9.57	12.05	5	5	5	50.9
Q88XU8	50S ribosomal protein L13	9.56	20.41	3	3	4	16.2
F9URQ9	Uncharacterized protein	9.31	6.00	4	4	4	76.2
F9UR54	PTS system, N-acetylglucosamine and glucose-specific EIICBA component	9.23	5.59	2	2	2	70.3
Q88YW8	50S ribosomal protein L10	8.80	30.54	3	3	3	17.9

F9UR64	NADH peroxidase	8.40	9.09	4	4	4	48.2
F9USY7	Maltodextrin-binding protein	8.23	6.47	2	2	3	45.6
F9UTA3	GMP reductase	7.81	6.79	2	2	3	39.9
F9UNV2	Bifunctional protein: transcription regulator sugar kinase, ROK family	7.74	11.56	3	3	3	33.7
F9UMJ0	Acetolactate synthase	7.67	13.93	6	6	7	61.2
Q88V89	Isoleucine--tRNA ligase	7.64	4.51	4	4	4	106.3
Q88UV8	D-alanine--D-alanine ligase	7.29	7.30	2	2	2	41.3
F9UPA5	Transpeptidase-transglycosylase (Penicillin binding protein 1A)	7.25	3.91	2	2	2	83.1
Q88XZ7	Serine--tRNA ligase 2	7.25	6.35	2	2	2	48.1
Q88XW0	DNA-directed RNA polymerase subunit alpha	7.16	9.55	3	3	3	34.8
Q88XU7	30S ribosomal protein S9	6.85	16.92	2	2	3	14.6
F9UN58	ATP-dependent Clp protease, ATP-binding subunit ClpE	6.66	3.79	2	2	2	81.7
Q88XW7	50S ribosomal protein L15	6.65	19.58	2	2	2	15.3
Q88YW7	50S ribosomal protein L7/L12	6.60	21.31	2	2	3	12.6
Q88XP6	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	6.32	6.33	3	3	3	53.2
Q88UX0	30S ribosomal protein S4	6.21	11.88	3	3	3	22.9
F9UTG0	Glutathione reductase	6.11	5.63	2	2	2	48.2
F9UMJ6	ATP-dependent Clp protease, ATP-binding subunit ClpC	6.11	3.82	2	2	2	92.6
Q88WZ5	UDP-N-acetylmuramate--L-alanine ligase	6.06	6.65	2	2	2	48.7
Q88XW9	30S ribosomal protein S5	6.00	13.25	2	2	2	17.3
F9UN64	Serine-type D-Ala-D-Ala carboxypeptidase	5.97	6.31	2	2	2	45.2
F9UPK1	ABC transporter, ATP-binding protein, ChvD family	5.84	4.25	2	2	2	71.3
F9UT53	Cystathionine beta-lyase / cystathionine gamma-lyase	5.78	6.30	2	2	2	40.8
F9UQC3	Cell division protein FtsZ	5.71	6.32	2	2	2	45.0
F9UL42	Homoserine dehydrogenase	5.53	5.37	2	2	2	46.6
P71479	Bifunctional protein PyrR 1	5.49	12.78	2	2	2	19.8
F9UP42	Acetyl-CoA carboxylase, biotin carboxylase subunit	5.36	4.55	2	2	2	50.7
Q88UX7	Valine--tRNA ligase	5.33	3.04	2	2	2	101.5
F9UPE6	Uncharacterized protein	5.30	12.70	2	2	2	25.3
F9UPH4	Transcription regulator, LysR family	5.15	7.21	2	2	2	36.8
Q88XB8	S-adenosylmethionine synthase	5.11	5.57	2	2	2	42.7
Q88X05	Foldase protein PrsA 1	5.06	7.05	2	2	2	32.6
F9UU97	Phosphoenolpyruvate carboxykinase (ATP)	5.05	4.52	2	2	2	61.8
F9UL89	Prophage P1 protein 2, mitogenic factor, cell surface lipoprotein	5.02	6.20	2	2	2	41.8
F9UQH9	Extracellular protein, DUF336 family	4.87	13.53	2	2	2	18.0
Q6LWD9	Nickase	4.84	2.48	2	2	2	80.0
Q88VQ7	Histidine--tRNA ligase	4.83	5.63	2	2	2	47.9
F9UQ85	Extracellular protein, cell-wall anchored	4.45	5.08	2	2	2	45.8
F9UQ00	Transcription termination/antitermination protein NusA	4.41	5.68	2	2	2	44.3
F9UMN7	NADH dehydrogenase, membrane-anchored	4.24	3.46	2	2	2	71.9
Q88VS3	Glycine--tRNA ligase beta subunit	4.22	3.31	2	2	2	78.5
F9UQZ2	Prophage P2a protein 4 AAA ATPase	4.12	6.70	4	4	4	66.5
F9URC3	Oligoendopeptidase F	4.03	3.51	3	3	3	67.2

F9UP07	Signal recognition particle protein	3.49	4.34	2	2	2	53.9
F9UQ97	Ribonuclease J	3.45	5.38	3	3	3	61.8
F9UNI1	GTP cyclohydrolase-2	2.44	8.91	3	3	3	43.6
Q88VM6	D-alanine--D-alanyl carrier protein ligase	2.07	10.24	4	4	4	56.0

Band # 3

Accession	Description	Score	Coverage	# Unique Peptides	# Peptides	# PSMs	MW [kDa]
Q88VE0	Elongation factor Tu	894.04	74.43	28	28	300	43.4
Q88YH5	Phosphoglycerate kinase	551.18	88.00	34	34	193	42.8
Q88YH3	Enolase 1	252.64	69.23	24	24	76	48.0
Q88UT5	Serine hydroxymethyltransferase	131.30	66.99	20	20	38	44.4
F9UNV9	Glutamine synthetase	123.12	61.38	26	26	43	50.9
Q88UI4	Glucose-6-phosphate isomerase	113.51	53.78	19	19	38	49.8
F9UM10	Glyceraldehyde 3-phosphate dehydrogenase	110.81	63.82	18	18	31	36.4
Q88WZ5	UDP-N-acetylmuramate--L-alanine ligase	105.30	54.36	22	22	34	48.7
F9UNI1	GTP cyclohydrolase-2	98.36	49.26	16	16	30	43.6
F9UN65	Aminotransferase	97.75	54.94	17	17	28	43.8
F9UTA5	Ribosome-binding ATPase YchF	96.84	75.14	20	20	32	39.9
F9UPM8	Peptidase T	96.58	50.00	13	13	28	45.1
Q88TV5	Tyrosine--tRNA ligase	88.32	53.35	17	17	28	47.0
Q88UV8	D-alanine--D-alanine ligase	86.27	60.54	17	17	28	41.3
P77884	Dihydroorotase	82.35	53.49	10	10	25	45.4
F9UPS7	RNA polymerase sigma factor SigA	81.87	47.01	17	17	25	41.4
Q88UT2	Peptide chain release factor 1	80.38	49.72	14	14	23	41.0
Q88XB8	S-adenosylmethionine synthase	78.83	45.82	16	16	25	42.7
F9UP39	3-oxoacyl-[acyl-carrier-protein] synthase 2	77.46	39.51	9	9	24	42.4
F9UPM3	Pyruvate kinase	75.61	42.32	19	19	24	62.8
Q88YE8	Phosphoglucosamine mutase	74.11	46.34	17	17	26	48.3
F9UL89	Prophage P1 protein 2, mitogenic factor, cell surface lipoprotein	64.53	53.49	14	14	19	41.8
F9UMW9	Glutamate dehydrogenase	63.87	45.98	14	14	19	48.6
Q88UU3	ATP synthase subunit beta	63.36	35.55	12	12	16	50.8
F9UTG0	Glutathione reductase	63.08	44.59	15	15	21	48.2
Q88Z54	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 2	61.57	46.95	12	12	19	45.8
F9UTW6	Glutathione reductase	61.49	41.31	14	14	23	47.3
F9UL68	Aminopeptidase C	60.07	39.50	13	13	21	50.2
F9UTR7	NADH dehydrogenase	55.36	38.00	11	11	19	43.8
F9UNU0	Endolytic murein transglycosylase	53.43	43.89	15	15	17	44.7
Q88VE2	ATP-dependent Clp protease ATP-binding subunit ClpX	53.39	45.61	14	14	17	46.5
F9UN44	Glutathione reductase	52.62	45.23	15	15	22	47.6
F9UTT2	Fructose-bisphosphate aldolase	50.92	48.78	11	11	16	30.9
F9US66	Serine protease HtrA	49.81	39.05	12	12	14	43.1
F9UR64	NADH peroxidase	49.27	52.55	15	15	19	48.2
Q88VZ6	GTPase Der	48.53	46.56	17	17	20	48.7
F9UNS5	6-phosphogluconate dehydrogenase, decarboxylating	47.28	37.03	14	14	15	52.9
Q88UX4	Probable tRNA sulfurtransferase	46.59	35.80	12	12	17	45.4

Q88V80	UDP-N-acetylmuramoylalanine--D-glutamate ligase	45.91	32.90	11	11	14	50.1
F9UL33	N-acetylglucosamine-6-phosphate deacetylase	44.38	34.92	10	10	15	41.4
F9USZ1	Alpha-amylase, maltodextrins and cyclomaltodextrins	44.09	33.86	12	12	14	49.9
F9UL42	Homoserine dehydrogenase	43.80	44.63	16	16	16	46.6
Q88WA8	Asparagine--tRNA ligase 2	40.37	36.34	13	13	15	49.8
F9UQS7	UDP-N-acetylmuramyl tripeptide synthase	40.31	32.14	12	12	16	49.7
F9UP42	Acetyl-CoA carboxylase, biotin carboxylase subunit	40.04	34.63	13	13	15	50.7
Q88VQ7	Histidine--tRNA ligase	38.93	41.78	13	13	16	47.9
Q88VJ4	30S ribosomal protein S2	37.10	40.82	10	10	13	30.2
Q88VJ2	D-lactate dehydrogenase	37.00	42.17	15	15	15	37.2
Q88TY1	Probable D-serine dehydratase	36.87	30.05	10	10	12	47.5
F9UPL0	30S ribosomal protein S1	36.49	25.41	12	12	17	47.1
F9UTR4	Acetate kinase	35.32	31.41	9	9	11	43.8
F9UTG2	Adenylosuccinate lyase	34.76	31.25	11	11	12	49.0
F9UQL8	Aspartokinase	34.08	23.11	7	7	9	49.8
Q88XZ2	DNA-directed RNA polymerase subunit beta'	32.00	7.25	8	8	10	135.2
F9UTP6	Glycolate dehydrogenase, subunit GlcD,FAD-binding	30.97	21.34	8	8	11	50.2
Q88YL5	Peptide chain release factor 2	30.53	30.50	9	9	12	42.6
P56512	L-lactate dehydrogenase 1	29.32	32.19	10	10	11	34.2
F9UNY1	Glycine betaine/carnitine/choline ABC transporter, ATP-binding protein	29.17	25.13	9	9	11	44.4
F9US34	Beta sliding clamp	28.80	28.23	10	10	12	41.5
F9UPX5	Protein DltD	28.46	20.00	6	6	8	48.6
F9UT87	Cyclopropane-fatty-acyl-phospholipid synthase	28.03	29.97	8	8	11	45.3
Q88XY3	50S ribosomal protein L2	27.66	40.86	9	9	13	30.2
F9UQ93	Pyruvate dehydrogenase complex, E1 component,alpha subunit	27.29	29.46	10	10	12	41.4
F9UU99	Glutamate decarboxylase	27.13	21.32	9	9	12	53.5
Q88YH4	Triosephosphate isomerase	27.02	32.14	7	7	10	27.0
F9UQ00	Transcription termination/antitermination protein NusA	26.49	21.73	7	7	9	44.3
Q88V24	N-acetyldiaminopimelate deacetylase	26.30	16.41	4	4	9	42.1
F9UKV7	Lipoprotein	26.03	21.65	6	6	8	34.3
F9UMS2	Fumarate reductase, flavoprotein subunit	25.76	19.70	6	6	7	48.7
Q88VJ5	Elongation factor Ts	25.18	32.53	8	8	11	31.6
F9ULL7	Aminopeptidase C	24.73	23.29	8	8	9	50.0
Q88UU1	ATP synthase subunit alpha	24.71	26.39	11	11	11	54.5
Q88SV6	Adenylosuccinate synthetase	24.67	22.14	7	7	8	47.2
Q890J4	Gamma-glutamyl phosphate reductase	24.06	25.24	8	8	10	44.6
Q88YW8	50S ribosomal protein L10	23.84	44.91	5	5	7	17.9
F9UTA3	GMP reductase	22.56	22.19	6	6	8	39.9
F9ULC7	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	22.05	23.94	9	9	9	44.4
Q88XY8	Elongation factor G	20.89	9.17	6	6	7	77.0
Q88Y74	GMP synthase [glutamine-hydrolyzing]	20.28	20.46	9	9	10	57.4
F9UR33	SAM-dependent methyltransferase	20.12	24.94	8	8	8	43.7
F9UQL7	Non-proteolytic protein, peptidase family M16	20.06	9.98	3	3	5	46.7
Q88YM5	60 kDa chaperonin	19.99	13.12	7	7	8	57.4

F9UUC0	Endopeptidase PepO	19.92	11.76	6	6	6	71.9
Q88YW9	50S ribosomal protein L1	18.51	34.93	6	6	6	24.8
F9UMC8	Alkaline shock protein	17.99	39.29	5	5	8	15.8
Q88UZ4	Protein RecA	17.22	18.16	6	6	7	40.6
F9UPK7	DNA-binding protein	16.44	36.26	2	2	5	9.6
P59603	Argininosuccinate synthase	16.25	17.76	7	7	8	45.1
Q88T35	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2	16.19	34.78	7	7	8	26.1
Q88UU5	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1	16.12	20.32	7	7	7	47.1
Q88W32	Probable manganese-dependent inorganic pyrophosphatase	15.89	21.04	5	5	5	33.6
F9UNV2	Bifunctional protein: transcription regulator sugar kinase, ROK family	15.86	20.63	5	5	5	33.7
F9ULY4	UTP--glucose-1-phosphate uridylyltransferase	15.75	20.26	4	4	5	34.2
F9UP45	Enoyl-[acyl-carrier-protein] reductase [NADH]	15.41	32.54	6	6	7	26.9
F9ULZ0	Phosphoglucomutase	15.41	12.70	6	6	7	63.5
Q88XY0	30S ribosomal protein S3	15.16	40.55	8	8	8	24.2
Q88XX4	50S ribosomal protein L5	15.10	37.78	5	5	6	20.2
Q88XY5	50S ribosomal protein L4	14.94	26.09	4	4	4	22.6
Q88Z28	Lysine--tRNA ligase	14.84	11.62	5	5	6	57.6
F9UMS1	Fumarate hydratase class II	14.52	11.30	5	5	6	49.5
Q88XW9	30S ribosomal protein S5	14.19	37.35	5	5	5	17.3
Q88Z31	ATP-dependent zinc metalloprotease FtsH	14.06	7.52	5	5	6	80.8
Q88XP7	Glutamyl-tRNA(Gln) amidotransferase subunit A	13.60	20.12	5	5	5	52.1
Q88WM7	Exodeoxyribonuclease 7 large subunit	13.60	14.54	6	6	6	50.5
F9UPA5	Transpeptidase-transglycosylase (Penicillin binding protein 1A)	13.51	6.65	4	4	4	83.1
F9UTE4	Uncharacterized protein	13.51	12.03	5	5	5	47.9
F9UQZ0	Prophage P2a protein 6 endonuclease	13.40	17.28	5	5	5	40.6
F9UTW4	Glycine betaine/carnitine/choline ABC transporter, substrate binding and permease protein	13.24	10.63	4	4	4	55.8
F9UQC4	Cell division protein FtsA	12.96	10.94	5	5	5	48.3
Q88VM1	Chaperone protein DnaJ	12.61	14.47	4	4	5	40.5
F9UQH6	Catabolite control protein A	12.46	15.48	4	4	5	36.3
F9UNL0	Cysteine desulfurase	12.46	12.62	5	5	5	44.9
Q88ZS1	Mannitol-1-phosphate 5-dehydrogenase	12.36	9.87	3	3	4	43.2
Q88VM0	Chaperone protein DnaK	12.34	8.04	4	4	4	66.7
F9UTG6	Cardiolipin synthase	12.31	10.10	4	4	4	55.7
F9UNK9	ABC transporter, iron-sulfur cluster assembly protein SufD	12.17	6.70	2	2	4	47.4
F9USY7	Maltodextrin-binding protein	12.15	9.11	3	3	4	45.6
Q88XZ7	Serine--tRNA ligase 2	12.07	11.76	4	4	4	48.1
F9UQP4	Universal stress protein	11.65	18.13	2	2	3	17.5
F9UQQ1	L-2-hydroxyisocaproate dehydrogenase	11.41	18.03	5	5	5	32.5
F9UQ85	Extracellular protein, cell-wall anchored	11.03	10.85	3	3	3	45.8
Q88XP4	Uncharacterized RNA methyltransferase lp_1151	10.99	10.72	3	3	4	51.0
F9UKZ5	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase	10.96	7.36	2	2	3	50.2
Q88W26	ATP-dependent protease ATPase subunit HslU	10.95	8.05	3	3	3	52.6
F9UTU2	Uncharacterized protein	10.89	7.22	2	2	3	43.7

F9UTV3	Multicopper oxidase	10.78	6.79	3	3	4	56.8
Q6LWG1	NADH oxidase (Putative)	10.53	5.15	2	2	3	50.6
F9URP6	6-phospho-beta-glucosidase	10.24	10.00	4	4	4	54.7
Q88VG4	GTPase Obg	10.10	11.37	2	3	3	46.7
F9UNS6	Recombination factor protein RarA ATPase, AAA family	10.08	6.77	2	2	3	50.9
Q88XY6	50S ribosomal protein L3	10.00	20.10	4	4	4	22.7
Q88XY9	30S ribosomal protein S7	9.77	32.69	4	4	4	17.8
F9UL91	Prophage P1 protein 5, superinfection exclusion (Cell surface N-anchored)	9.55	14.37	4	4	4	37.9
F9UQB3	Cysteine desulfurase	9.23	13.73	4	4	4	42.3
F9UPK6	Uncharacterized protein	9.12	7.11	4	4	5	48.8
F9UL45	PTS system, mannose-specific EIIAB component	8.80	9.88	3	3	3	35.3
F9UP94	Aminotransferase	8.54	9.48	3	3	3	43.8
Q88VS2	Glycine--tRNA ligase alpha subunit	8.43	14.05	3	3	3	34.5
F9URK6	NADPH-dependent FMN reductase family protein	8.23	11.14	4	4	4	47.4
F9UMN8	Lipoprotein, FMN-binding protein	8.20	21.43	3	3	3	32.9
Q88V96	tRNA-specific 2-thiouridylase MnmA	7.91	8.02	2	2	2	42.0
Q88XX1	50S ribosomal protein L6	7.83	18.54	3	3	3	19.3
Q88Z33	tRNA(Ile)-lysidine synthase	7.65	8.26	3	3	3	51.0
Q88XU8	50S ribosomal protein L13	7.40	26.53	3	3	3	16.2
Q88WU9	Threonine--tRNA ligase	7.34	3.98	2	2	2	73.8
Q88XW0	DNA-directed RNA polymerase subunit alpha	7.12	7.96	3	3	3	34.8
Q6LWD9	Nickase	7.07	3.94	2	2	2	80.0
F9UPH4	Transcription regulator, LysR family	6.92	11.60	3	3	3	36.8
Q88Z97	Methionine--tRNA ligase	6.71	3.95	2	2	2	76.7
F9UQJ7	DEAD-box ATP-dependent RNA helicase CshB	6.62	5.82	3	3	3	50.6
Q88YH9	ATP-dependent Clp protease proteolytic subunit	6.51	12.76	2	2	2	21.5
F9UT98	Serine-type D-Ala-D-Ala carboxypeptidase	6.50	8.78	3	3	4	46.9
F9UP44	Acetyl-CoA carboxylase, carboxyl transferase subunit alpha	6.24	9.77	2	2	2	27.6
Q88V10	Alanine--tRNA ligase	6.22	3.07	2	2	3	97.2
F9UM08	DNA-directed RNA polymerase, sigma factor 54	6.09	9.21	4	4	5	51.3
F9URG7	Glucose-6-phosphate 1-dehydrogenase	6.05	6.07	2	2	2	56.8
F9UN64	Serine-type D-Ala-D-Ala carboxypeptidase	6.00	8.25	3	3	3	45.2
Q88Z86	Bifunctional protein GlmU	5.93	10.43	3	3	3	50.0
Q88XZ0	30S ribosomal protein S12	5.81	18.25	2	2	2	15.2
F9UQM2	Site-determining protein	5.75	10.45	2	2	2	29.1
Q88Z84	Ribose-phosphate pyrophosphokinase 1	5.63	8.90	3	3	3	35.9
Q88VY1	ATP-dependent 6-phosphofructokinase	5.55	6.88	2	2	2	34.2
Q88YY0	Glutamate--tRNA ligase	5.50	4.44	2	2	2	56.9
Q88YW7	50S ribosomal protein L7/L12	5.43	18.85	2	2	2	12.6
Q88ZJ6	Spermidine/putrescine import ATP-binding protein PotA	5.19	6.30	2	2	3	40.7
Q88VM6	D-alanine--D-alanyl carrier protein ligase	5.08	10.04	4	4	4	56.0
F9UMF3	Dipeptidase	4.95	4.91	2	2	2	52.3
Q88YE7	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	4.76	3.64	2	2	2	65.4
F9UMY6	dTDP-4-dehydrorhamnose 3,5-epimerase	4.71	22.28	3	3	3	21.8

Q88UX0	30S ribosomal protein S4	4.70	18.32	4	4	4	22.9
F9UP59	Cyclopropane-fatty-acyl-phospholipid synthase	4.67	5.13	2	2	2	44.7
Q88XU7	30S ribosomal protein S9	4.67	16.92	2	2	2	14.6
F9UPB8	Glycosyl hydrolase, family 25	4.64	12.98	4	4	4	50.0
F9UU91	Cell surface protein, CscB family	4.61	0.97	2	2	2	118.7
F9UQH9	Extracellular protein, DUF336 family	4.58	13.53	2	2	2	18.0
Q88XY1	50S ribosomal protein L22	4.30	14.78	2	2	2	12.5
F9UNF9	Aminotransferase (PLP-dependent) with N-terminal regulator domain, GntR family	4.10	4.55	2	2	2	54.1
F9UP37	Malonyl CoA-acyl carrier protein transacylase	4.05	6.77	2	2	2	33.3
Q88VQ8	Aspartate--tRNA ligase	3.94	7.02	5	5	5	67.6
F9UMX4	UDP-galactopyranose mutase	3.75	5.36	2	2	2	43.2
F9UP82	23S rRNA methylase	3.53	8.13	3	3	3	50.3
Q06115	Choloylglycine hydrolase	3.28	7.10	2	2	2	37.0
Q88W02	CCA-adding enzyme	2.66	6.88	2	2	2	45.1
F9USJ3	Extracellular protein	2.45	9.49	2	2	2	29.3
Q88X05	Foldase protein PrsA 1	2.39	5.70	2	2	2	32.6
F9ULW6	Ribosome hibernation promoting factor	2.19	20.74	2	2	2	21.7
Q88UX7	Valine--tRNA ligase	1.87	2.14	2	2	2	101.5
Q9RE01	Uracil phosphoribosyltransferase	1.61	7.66	2	2	2	23.0
Q88VE1	Trigger factor	0.00	4.77	2	2	2	49.4

Band # 4

Accession	Description	Score	Coverage	# Unique Peptides	# Peptides	# PSMs	MW [kDa]
F9UM10	Glyceraldehyde 3-phosphate dehydrogenase	776.26	80.29	29	29	244	36.4
Q88VJ2	D-lactate dehydrogenase	221.28	72.89	28	28	83	37.2
Q88UU2	ATP synthase gamma chain	158.76	61.46	20	20	52	34.5
F9UTA3	GMP reductase	143.70	80.16	21	21	46	39.9
Q88VE0	Elongation factor Tu	136.55	64.30	19	19	45	43.4
F9UT17	Acetate kinase	134.36	73.67	20	20	38	42.9
F9UT87	Cyclopropane-fatty-acyl-phospholipid synthase	123.72	51.89	18	18	36	45.3
F9UR85	Aspartate-semialdehyde dehydrogenase	108.93	60.34	17	17	34	38.2
F9ULC7	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	107.72	75.56	26	26	37	44.4
Q88XW0	DNA-directed RNA polymerase subunit alpha	102.20	43.95	15	15	38	34.8
F9UTI2	Dihydropteroate synthase	98.06	53.66	18	18	39	43.4
F9UMU0	Oxidoreductase, NAD(P)-dependent	97.28	60.80	14	14	32	35.9
Q88WK2	Phosphate acyltransferase	96.49	41.95	12	12	27	37.4
F9UMW1	Oxidoreductase, NAD-dependent	91.02	61.49	14	14	26	37.1
Q88YH5	Phosphoglycerate kinase	88.18	65.25	23	23	33	42.8
F9UMX1	UDP-N-acetylglucosamine 2-epimerase	86.03	61.83	13	13	31	41.2
F9UP94	Aminotransferase	84.50	35.66	11	11	24	43.8
F9UMX4	UDP-galactopyranose mutase	84.15	41.02	12	12	24	43.2
F9UMU4	Formate-dependent phosphoribosylglycinamide formyltransferase	82.96	39.85	11	11	26	42.8
Q88V04	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	82.94	56.98	15	15	29	39.0

F9UN64	Serine-type D-Ala-D-Ala carboxypeptidase	82.52	52.91	19	19	29	45.2
P56512	L-lactate dehydrogenase 1	80.57	45.94	18	18	26	34.2
F9UMH0	Aspartokinase	76.01	46.75	14	14	27	42.8
Q88WR3	Phenylalanine--tRNA ligase alpha subunit	75.79	53.45	20	20	29	39.3
Q88YH3	Enolase 1	75.57	41.40	13	13	23	48.0
F9UQV7	Prophage P2a protein 40 major capsid protein	75.52	59.66	15	15	24	38.1
F9UQZ0	Prophage P2a protein 6 endonuclease	71.82	52.41	20	20	28	40.6
Q88VJ4	30S ribosomal protein S2	68.65	58.05	17	17	26	30.2
F9UPA9	RNA methyltransferase	65.05	42.26	14	14	22	43.1
O08445	Alanine racemase	64.98	35.20	12	12	22	40.7
Q88W32	Probable manganese-dependent inorganic pyrophosphatase	64.63	44.98	14	14	19	33.6
F9UU32	3-hydroxy-3-methylglutaryl coenzyme A reductase	64.20	45.75	13	13	20	45.5
Q88VJ5	Elongation factor Ts	63.40	54.45	13	13	20	31.6
F9UQS9	Mannose-6-phosphate isomerase	62.90	52.96	12	12	21	35.9
F9UQN3	Cysteine desulfurase	62.81	47.40	13	13	20	42.3
F9UTT2	Fructose-bisphosphate aldolase	62.39	48.78	11	11	21	30.9
Q88V96	tRNA-specific 2-thiouridylase MnmA	62.30	67.11	20	20	27	42.0
Q88VM1	Chaperone protein DnaJ	57.54	53.68	16	16	23	40.5
F9UNI1	GTP cyclohydrolase-2	56.90	35.15	13	13	18	43.6
F9ULY4	UTP--glucose-1-phosphate uridylyltransferase	56.50	62.09	14	14	20	34.2
F9USY0	Dihydroxyacetone phosphotransferase,dihydroxyacetone binding subunit	55.54	36.14	11	11	21	36.1
F9UQH7	Xaa-Pro dipeptidase	54.82	36.59	12	12	25	41.1
Q88V05	Queuine tRNA-ribosyltransferase	53.77	38.68	10	10	16	42.8
F9US66	Serine protease HtrA	53.74	44.76	12	12	15	43.1
F9UMV1	Diacylglycerol kinase family protein	51.52	35.00	11	11	21	37.0
F9UT98	Serine-type D-Ala-D-Ala carboxypeptidase	50.95	41.67	14	14	19	46.9
F9UU20	Tryptophan-tRNA synthetase	50.19	42.90	11	11	19	37.6
F9USH3	1,3-propanediol dehydrogenase	48.41	42.82	13	13	17	42.1
F9UT53	Cystathionine beta-lyase / cystathionine gamma-lyase	47.38	37.80	10	10	17	40.8
F9UPM3	Pyruvate kinase	46.98	36.01	16	16	18	62.8
F9URL2	L-asparaginase	46.21	36.73	8	8	15	34.4
F9UL91	Prophage P1 protein 5, superinfection exclusion (Cell surface N-anchored)	45.91	37.07	12	12	17	37.9
Q88ZS1	Mannitol-1-phosphate 5-dehydrogenase	45.67	49.35	14	14	15	43.2
F9UNG8	Metal-dependent phosphohydrolase, HD family	44.67	43.87	13	13	18	37.0
F9UMY7	dTDP-glucose 4,6-dehydratase	44.02	39.77	12	12	18	38.6
F9UT86	Cell surface protein, membrane-anchored	43.76	47.83	8	8	11	38.0
F9UNR3	Ribosome biogenesis GTPase, YqeH family	43.51	50.26	16	16	17	41.7
Q88UV8	D-alanine--D-alanine ligase	43.50	40.00	12	12	14	41.3
F9UMU7	Lipoprotein, pheromone	43.34	45.78	13	13	14	42.6
F9UQS3	Threonylcarbamoyl-AMP synthase	41.90	46.61	11	11	15	36.3
F9UTR4	Acetate kinase	41.25	31.41	9	9	12	43.8
P77885	Carbamoyl-phosphate synthase pyrimidine-specific small chain	40.00	35.99	9	9	15	40.0
Q88V81	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide)	38.98	32.51	10	10	14	38.7

	pyrophosphoryl-undecaprenol N-acetylglucosamine transferase						
F9UP59	Cyclopropane-fatty-acyl-phospholipid synthase	38.59	26.67	9	9	14	44.7
F9UPC4	Oxidoreductase, FAD-dependent	37.85	25.82	9	9	14	40.4
F9UMC8	Alkaline shock protein	37.85	45.71	6	6	12	15.8
F9UPY0	Serine-type D-Ala-D-Ala carboxypeptidase	37.31	35.04	12	12	15	43.3
F9UQM6	Cell shape determining protein MreB	36.15	38.92	10	10	13	35.1
Q88XY3	50S ribosomal protein L2	35.77	53.76	12	12	19	30.2
F9UM22	Polar amino acid ABC transporter, substrate binding and permease protein	35.65	19.25	7	7	11	52.2
F9UPH4	Transcription regulator, LysR family	35.27	39.18	11	11	13	36.8
F9UP39	3-oxoacyl-[acyl-carrier-protein] synthase 2	35.16	30.24	7	7	11	42.4
Q88YM5	60 kDa chaperonin	35.08	29.39	13	13	14	57.4
F9USG7	Aminohydrolase/peptidase, M20D family	34.75	29.57	7	7	11	43.0
F9UP45	Enoyl-[acyl-carrier-protein] reductase [NADH]	34.70	44.05	9	9	15	26.9
F9UNY1	Glycine betaine/carnitine/choline ABC transporter, ATP-binding protein	34.65	32.16	12	12	16	44.4
F9UL33	N-acetylglucosamine-6-phosphate deacetylase	34.35	28.04	10	10	15	41.4
F9UL45	PTS system, mannose-specific EIIAB component	33.81	29.63	9	9	12	35.3
Q88T35	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2	33.61	38.26	9	9	16	26.1
F9URM4	Cystathionine beta-lyase	33.36	27.64	10	10	11	45.4
F9UNS5	6-phosphogluconate dehydrogenase, decarboxylating	33.30	31.80	11	11	11	52.9
F9ULY7	Thioredoxin reductase	33.25	47.12	10	10	10	33.4
F9UN65	Aminotransferase	33.10	36.20	10	10	12	43.8
F9UTR7	NADH dehydrogenase	33.01	27.75	9	9	13	43.8
Q88WU9	Threonine--tRNA ligase	32.79	19.42	9	9	11	73.8
F9UQ72	DNA-directed DNA polymerase III, delta chain	32.63	38.81	11	11	11	39.7
F9UNG1	Aminotransferase	32.53	29.76	9	9	11	41.6
F9UNV9	Glutamine synthetase	31.68	35.04	11	11	12	50.9
F9UQB3	Cysteine desulfurase	31.40	20.47	6	6	9	42.3
Q88YW9	50S ribosomal protein L1	31.15	36.68	9	9	12	24.8
Q88VM0	Chaperone protein DnaK	30.79	17.68	8	8	10	66.7
Q88UH9	UPF0210 protein lp_2507	30.42	20.81	9	9	11	46.6
Q88YH4	Triosephosphate isomerase	29.28	49.21	10	10	12	27.0
F9USZ2	Maltodextrin ABC transporter, ATP-binding protein	29.27	26.09	7	7	9	41.3
Q88WA8	Asparagine--tRNA ligase 2	29.17	26.39	9	9	11	49.8
F9UL89	Prophage P1 protein 2, mitogenic factor, cell surface lipoprotein	28.48	30.49	10	10	12	41.8
F9UUD1	ATPase, MoxR family	26.59	25.19	7	7	8	43.9
Q88UV2	Methionine import ATP-binding protein MetN 2	26.55	37.61	10	10	10	37.7
Q88UI4	Glucose-6-phosphate isomerase	26.38	28.44	10	10	11	49.8
F9UM27	Phosphate acetyltransferase	25.16	29.54	8	8	12	34.4
F9UPP5	Succinyl-diaminopimelate desuccinylase	24.15	23.10	7	7	8	40.8
Q88XX4	50S ribosomal protein L5	23.56	47.22	8	8	10	20.2
Q88XY9	30S ribosomal protein S7	23.54	57.05	7	7	8	17.8
F9USW3	NADH:flavin oxidoreductase/NADH oxidase	23.52	32.79	9	9	10	41.0

Q88YW8	50S ribosomal protein L10	23.38	65.87	8	8	8	17.9
Q88YW7	50S ribosomal protein L7/L12	23.03	50.82	6	6	8	12.6
Q88XY8	Elongation factor G	22.83	16.48	9	9	10	77.0
F9UP44	Acetyl-CoA carboxylase, carboxyl transferase subunit alpha	22.64	35.16	5	5	7	27.6
F9URN7	Uncharacterized protein	22.54	23.97	9	9	10	40.7
Q88XY6	50S ribosomal protein L3	21.57	32.54	6	6	8	22.7
F9UMW9	Glutamate dehydrogenase	21.10	18.75	6	6	7	48.6
F9ULD9	ATP-dependent Clp protease, ATP-binding subunit ClpL	20.69	13.92	8	8	8	77.7
Q88Z84	Ribose-phosphate pyrophosphokinase 1	20.42	23.93	9	9	10	35.9
F9UR64	NADH peroxidase	20.40	13.30	5	5	8	48.2
F9UMR6	Malic enzyme, NAD-dependent	19.78	19.95	6	6	9	40.1
F9UPS7	RNA polymerase sigma factor SigA	19.40	18.75	7	7	8	41.4
F9UND1	Extracellular protein, membrane-anchored	19.29	16.57	5	5	7	40.1
F9UTU1	LL-diaminopimelate aminotransferase, AAT family, PLP-dependent	19.26	27.62	7	7	7	42.8
F9UTB1	Pyrimidine nucleoside transport protein	19.19	14.75	4	4	6	46.3
F9UL63	Transcriptional attenuator, cell envelope-related, LytR family	18.73	19.65	6	6	7	43.6
Q88WL3	Methionyl-tRNA formyltransferase	18.65	23.97	7	7	8	34.4
Q88XW9	30S ribosomal protein S5	18.61	48.80	7	7	8	17.3
Q88V03	Holliday junction ATP-dependent DNA helicase RuvB	18.22	23.51	6	6	6	37.3
F9UMV3	Transcription regulator, TetR family	18.19	37.17	6	6	7	21.6
F9UQB9	Cell division initiation protein DivIVA	18.08	24.14	6	6	7	26.2
Q88Y74	GMP synthase [glutamine-hydrolyzing]	17.75	16.22	8	8	8	57.4
F9UMD7	Type I restriction-modification system,specificity subunit	17.54	17.11	6	6	7	42.3
F9UPM8	Peptidase T	17.21	16.50	5	5	6	45.1
Q88Z14	NH(3)-dependent NAD(+) synthetase	17.06	26.55	5	5	5	30.6
F9UKZ0	Uncharacterized protein	17.04	24.60	6	6	6	42.5
F9UR33	SAM-dependent methyltransferase	16.88	23.92	8	8	8	43.7
F9UQT2	Branched-chain amino acid aminotransferase	16.82	22.51	5	5	6	37.9
F9ULE0	NAD-independent L-lactate dehydrogenase	16.58	19.67	5	5	5	38.7
Q88V33	Probable transcriptional regulatory protein lp_2253	16.53	21.90	5	5	6	26.4
Q88XY0	30S ribosomal protein S3	16.21	35.48	8	8	8	24.2
F9UU82	2,5 diketo-D-gluconic acid-like reductase, NADP dependent (Promiscuous)	15.99	29.37	7	7	8	31.7
F9UPK7	DNA-binding protein	15.83	67.03	4	4	5	9.6
F9UQ21	Hydroxymethylglutaryl-CoA synthase	15.45	19.02	6	6	6	42.8
F9UT85	Xylose operon regulator, ROK family	15.27	19.01	5	5	5	42.7
Q88UU3	ATP synthase subunit beta	15.08	11.78	4	4	4	50.8
Q88VY1	ATP-dependent 6-phosphofructokinase	15.04	20.31	6	6	6	34.2
F9UUH6	PTS-associated protein	15.01	21.82	6	6	6	41.0
Q88XY5	50S ribosomal protein L4	14.78	30.43	5	5	5	22.6
F9UTL0	Glutamine-fructose-6-phosphate transaminase (Isomerizing)	14.70	20.77	5	5	5	40.5
F9UTA7	Chomosome partitioning protein, DNA-binding protein	14.49	22.64	6	6	6	33.1
Q88XU8	50S ribosomal protein L13	14.49	34.01	4	4	5	16.2
F9UQ92	Pyruvate dehydrogenase complex, E1 component,beta subunit	13.81	17.23	5	5	6	35.4
Q88X05	Foldase protein PrsA 1	13.78	16.44	4	4	4	32.6

Q88WG0	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta 2	13.65	23.49	5	5	5	30.9
Q88UU0	ATP synthase subunit delta	13.56	28.73	4	4	5	20.0
F9UNX0	Xaa-Pro aminopeptidase	13.43	18.98	5	5	5	37.8
F9UPI8	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (Cobalamine-independent methonine synthase)	13.36	18.85	6	6	7	43.2
F9UQH6	Catabolite control protein A	13.36	17.56	5	5	7	36.3
F9UMN8	Lipoprotein, FMN-binding protein	13.33	16.88	4	4	4	32.9
F9UMI5	Transcriptional attenuator, cell envelope-related, LytR family	13.32	15.36	4	4	5	37.7
Q88VS2	Glycine--tRNA ligase alpha subunit	13.20	22.07	5	5	5	34.5
F9UTF3	DegV family protein	13.17	15.41	3	3	4	29.6
F9UPL0	30S ribosomal protein S1	13.04	23.54	10	10	10	47.1
F9URX3	Alcohol dehydrogenase, zinc-binding	12.93	21.39	6	6	8	36.7
F9UQH2	Ribokinase	12.78	16.01	4	4	4	31.7
Q88W73	Carbamoyl-phosphate synthase small chain	12.74	9.72	3	3	4	39.2
Q88Z76	CTP synthase	12.62	13.59	6	6	6	59.7
P77883	Aspartate carbamoyltransferase	12.37	19.29	4	4	4	34.7
F9UTG0	Glutathione reductase	12.31	8.33	3	3	4	48.2
F9UMR5	Citrate lyase regulator, SorC family	12.26	15.31	4	4	4	35.6
F9UNV2	Bifunctional protein: transcription regulator sugar kinase, ROK family	12.02	19.06	5	5	5	33.7
F9UPX5	Protein DltD	11.94	17.18	5	5	5	48.6
F9URN8	Acetyltransferase, GNAT family	11.64	12.21	4	4	4	44.7
Q88ZH4	Teichoic acids export ATP-binding protein TagH	11.58	11.57	4	4	5	40.8
Q88V41	Acetate kinase	11.45	22.42	7	7	7	43.4
F9UMX6	Glycosyltransferase, family 1 (GT1)	11.37	27.20	7	7	8	40.2
Q88TV5	Tyrosine--tRNA ligase	11.34	10.05	4	4	4	47.0
F9UL68	Aminopeptidase C	11.22	15.12	5	5	5	50.2
Q88WT1	UPF0348 protein lp_1534	11.07	9.40	3	3	4	43.0
Q88YY0	Glutamate--tRNA ligase	11.02	9.27	4	4	4	56.9
Q88UC0	Ferredoxin--NADP reductase	10.99	13.60	4	4	4	36.1
Q88YL5	Peptide chain release factor 2	10.97	11.14	3	3	3	42.6
F9UPH8	Aldose 1-epimerase family protein	10.95	14.73	3	3	4	32.7
F9ULW6	Ribosome hibernation promoting factor	10.89	31.38	4	4	5	21.7
Q88XX2	30S ribosomal protein S8	10.86	23.66	3	3	4	14.6
Q88Y39	Aspartate--ammonia ligase	10.74	19.88	5	5	5	37.5
F9UN61	1,2-diacylglycerol 3-glucosyltransferase	10.74	18.11	6	6	6	44.2
F9UL10	Cell surface protein, ErfK/YbiS/YcfS/YnhG family	10.69	7.97	3	3	5	51.1
Q88YE8	Phosphoglucosamine mutase	10.48	9.09	3	3	3	48.3
Q88YM8	Redox-sensing transcriptional repressor Rex	10.30	22.22	5	5	5	25.2
Q88XW7	50S ribosomal protein L15	10.24	27.97	3	3	3	15.3
Q88UX4	Probable tRNA sulfurtransferase	10.06	10.86	4	4	4	45.4
F9UQH9	Extracellular protein, DUF336 family	9.92	25.88	4	4	4	18.0
F9UNL3	Iron chelatin ABC transporter, substrate binding protein	9.91	13.20	3	3	3	38.6
Q88VQ7	Histidine--tRNA ligase	9.80	8.92	3	3	4	47.9
F9UP42	Acetyl-CoA carboxylase, biotin carboxylase subunit	9.80	11.26	4	4	4	50.7
Q88XY4	50S ribosomal protein L23	9.54	43.30	3	3	3	11.1
F9UTA5	Ribosome-binding ATPase YchF	9.50	13.93	4	4	4	39.9
F9URD4	Extracellular protein	9.17	15.91	3	3	3	23.1

F9UPC9	Pseudouridine synthase	9.12	12.05	3	3	3	33.8
Q88UX0	30S ribosomal protein S4	9.11	18.32	4	4	5	22.9
F9UP86	Aldose 1-epimerase	9.01	21.07	5	5	6	37.8
F9UQQ1	L-2-hydroxyisocaproate dehydrogenase	8.98	12.13	3	3	4	32.5
F9UMC9	Alkaline shock protein	8.96	20.55	2	2	3	16.1
F9UKV7	Lipoprotein	8.83	10.37	3	3	3	34.3
F9USY7	Maltodextrin-binding protein	8.78	8.63	3	3	4	45.6
F9UL47	PTS system, mannose-specific EIID component	8.70	11.48	3	3	3	33.6
F9ULZ0	Phosphoglucomutase	8.61	8.70	4	4	5	63.5
P77884	Dihydroorotase	8.36	10.00	3	3	3	45.4
F9USN8	DNA-binding ferritin-like protein, DPS family	8.34	21.94	3	3	3	18.0
F9UM03	Putative gluconeogenesis factor	8.29	10.81	2	2	2	36.6
F9UMN9	FAD:protein FMN transferase	8.26	10.83	4	4	4	43.4
Q88WZ5	UDP-N-acetylmuramate--L-alanine ligase	8.22	8.72	3	3	3	48.7
F9UL42	Homoserine dehydrogenase	8.22	8.88	3	3	3	46.6
F9UPJ8	DegV family protein	8.21	13.78	3	3	3	30.1
F9UTW6	Glutathione reductase	7.96	8.35	3	3	3	47.3
F9UUF4	Transcription regulator. LacI family, raffinose related	7.92	10.27	3	3	3	37.1
Q88WU6	50S ribosomal protein L20	7.76	24.58	3	3	3	13.6
F9UM84	Glutamine ABC transporter, ATP-binding protein	7.58	18.29	4	4	4	27.4
Q88XB8	S-adenosylmethionine synthase	7.56	7.34	2	2	2	42.7
Q890K5	DNA replication and repair protein RecF	7.32	12.03	4	4	4	42.4
Q88XW5	Adenylate kinase	7.24	25.57	4	4	4	24.4
F9UN23	Mannose-specific adhesin, LPXTG-motif cell wall anchor	7.17	3.56	2	2	2	107.9
F9US18	Anaerobic ribonucleoside-triphosphate reductase	7.06	4.73	3	3	3	83.7
F9UQ85	Extracellular protein, cell-wall anchored	6.99	8.55	3	3	3	45.8
F9USJ3	Extracellular protein	6.98	9.85	2	2	2	29.3
Q88XZ2	DNA-directed RNA polymerase subunit beta'	6.97	2.14	2	2	2	135.2
Q88XV9	50S ribosomal protein L17	6.88	19.69	2	2	2	14.2
F9UTQ0	Transport protein, MMPL family	6.82	2.61	2	2	2	143.2
F9UTV3	Multicopper oxidase	6.76	7.98	2	2	2	56.8
Q88WG9	3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ	6.70	14.97	2	2	2	16.0
F9UMH1	Asparagine synthase (Glutamine-hydrolysing)	6.68	5.37	2	2	2	73.1
Q88XZ0	30S ribosomal protein S12	6.58	23.36	4	4	4	15.2
P71479	Bifunctional protein PyrR 1	6.46	12.22	2	2	3	19.8
F9UQS7	UDP-N-acetylmuramyl tripeptide synthase	6.27	9.60	4	4	4	49.7
F9UN60	Phosphoenolpyruvate-protein phosphotransferase	6.22	4.34	2	2	2	63.1
F9UQ93	Pyruvate dehydrogenase complex, E1 component,alpha subunit	6.18	8.92	3	3	3	41.4
Q88YH9	ATP-dependent Clp protease proteolytic subunit	6.14	12.76	2	2	2	21.5
F9UU93	Cell surface protein, CscB family	6.11	8.55	2	2	2	27.4
F9UNW0	Uncharacterized protein	6.10	9.83	2	2	2	34.5
Q88YK3	HPr kinase/phosphorylase	6.08	10.79	3	3	3	34.8
F9UP38	3-oxoacyl-[acyl-carrier protein] reductase	6.00	12.40	2	2	2	25.7
Q88UZ3	Putative competence-damage inducible protein	5.87	5.71	2	2	2	45.0

F9URD2	Cysteine gamma synthase/O-succinylhomoserine (Thiol)-lyase	5.85	8.04	2	2	3	40.5
Q06115	Choloylglycine hydrolase	5.82	6.79	2	2	2	37.0
F9UPL2	Extracellular protein, with LysM peptidoglycan binding domain	5.76	17.62	3	3	3	21.6
Q88VA5	Putative ribose-phosphate pyrophosphokinase 2	5.68	7.17	2	2	3	35.0
F9UM66	Proline iminopeptidase	5.59	10.26	2	2	2	34.5
Q88Z86	Bifunctional protein GlmU	5.22	5.00	2	2	2	50.0
F9ULK7	Ribokinase	5.20	12.75	3	3	3	32.0
Q88VE1	Trigger factor	5.12	7.05	3	3	4	49.4
Q88XX1	50S ribosomal protein L6	4.96	10.67	2	2	2	19.3
F9ULM5	Single stranded DNA-binding protein	4.89	9.94	4	4	4	34.2
F9UQQ9	Cell shape determining protein MreB	4.88	6.61	2	2	2	35.8
Q88V80	UDP-N-acetylmuramoylalanine--D-glutamate ligase	4.86	9.37	3	3	3	50.1
Q88VL9	Protein GrpE	4.81	11.56	2	2	2	21.4
Q88XW2	30S ribosomal protein S13	4.79	14.05	2	2	2	13.6
Q88U67	Probable phosphoketolase 1	4.70	2.16	2	2	2	88.7
Q88WU8	Translation initiation factor IF-3	4.67	13.29	2	2	3	19.6
F9UN90	Polysaccharide export protein	4.62	4.50	2	2	2	61.4
F9ULT0	Ribonucleoside-diphosphate reductase	4.55	3.61	2	2	2	82.1
Q88XP7	Glutamyl-tRNA(Gln) amidotransferase subunit A	4.47	5.34	2	2	2	52.1
Q88XX9	50S ribosomal protein L16	4.46	17.36	2	2	2	16.1
F9UT64	Glycerol-3-phosphate cytidylyltransferase	4.25	12.69	2	2	2	15.8
Q88ZJ6	Spermidine/putrescine import ATP-binding protein PotA	4.06	7.12	2	2	2	40.7
F9UPJ4	Carboxy-terminal proteinase, S41 family,peptidoglycan-bound	4.04	4.07	2	2	2	53.2
F9UL73	ATPase, PilT family	3.93	8.95	3	3	5	44.3
F9UQP4	Universal stress protein	3.50	20.63	2	2	2	17.5
Q88SE8	Galactokinase	3.49	10.59	4	4	4	42.6
F9USG8	Short-chain dehydrogenase/oxidoreductase,classical SDR family, subgroup 1	3.47	7.77	2	2	2	31.5
F9UQ58	Glutamine ABC transporter, substrate binding and permease protein	3.13	4.89	2	2	2	53.8
Q88VQ8	Aspartate--tRNA ligase	2.91	6.69	4	4	4	67.6
Q88XY7	30S ribosomal protein S10	2.90	21.57	2	2	2	11.7
Q88XZ7	Serine--tRNA ligase 2	2.85	5.41	2	2	2	48.1
F9UUF3	Aldose 1-epimerase	2.78	6.98	2	2	2	38.3
F9UMY1	Glycosyltransferase, family 1 (GT1)	2.72	6.91	2	2	2	43.2
F9UQM5	Cell shape-determining protein MreC	2.53	8.54	2	2	2	30.1
Q88YK1	Glycerol-3-phosphate dehydrogenase [NAD(P)+]	2.52	10.95	2	2	2	36.6
F9UM05	Oligopeptide ABC transporter, substrate binding protein	2.49	5.09	2	2	2	61.0
F9UTJ4	Beta-lactamase family protein	2.48	9.12	3	3	3	37.3
Q88XU7	30S ribosomal protein S9	2.44	16.92	2	2	2	14.6
Q88XQ4	Xanthine phosphoribosyltransferase	2.41	17.44	3	3	3	21.4
F9USC2	Manganese transport protein	2.41	4.53	2	2	2	57.4
Q88XX6	50S ribosomal protein L14	2.18	16.39	2	2	2	13.1
F9UPA5	Transpeptidase-transglycosylase (Penicillin binding protein 1A)	2.17	2.74	2	2	2	83.1
F9ULS9	Ribonucleoside-diphosphate reductase, beta chain	2.17	7.14	3	3	3	39.1
Q88X53	Arginine--tRNA ligase	2.14	5.34	3	3	3	62.9

F9UTB5	Cystine ABC transporter, substrate binding protein	2.03	6.37	2	2	2	29.0
F9UPK2	Uncharacterized protein	1.90	14.00	2	2	2	17.1
Q88YI4	Nucleotide-binding protein lp_0779	1.89	5.44	2	2	2	33.3
Q88V24	N-acetyldiaminopimelate deacetylase	1.80	5.47	2	2	2	42.1
Q88VM6	D-alanine--D-alanyl carrier protein ligase	0.00	6.89	3	3	3	56.0
Q88VJ6	Uridylate kinase	0.00	12.92	2	2	2	25.9
F9UMF8	Pseudouridine synthase	0.00	7.85	2	2	2	32.3

Band # 5

Accession	Description	Score	Coverage	# Unique Peptides	# Peptides	# PSMs	MW [kDa]
Q88VM0	Chaperone protein DnaK	438.36	70.90	37	37	141	66.7
Q88WU9	Threonine--tRNA ligase	291.74	67.74	38	38	103	73.8
F9ULD9	ATP-dependent Clp protease, ATP-binding subunit ClpL	240.46	62.93	33	33	69	77.7
F9UNZ9	Phosphatase, dihydroxyacetone kinase family	192.32	68.66	29	29	59	60.7
F9UPM3	Pyruvate kinase	178.75	60.92	29	29	56	62.8
Q88Z97	Methionine--tRNA ligase	161.03	54.61	31	31	54	76.7
Q88VE0	Elongation factor Tu	158.71	64.56	20	20	51	43.4
Q88YM5	60 kDa chaperonin	145.20	65.43	30	30	46	57.4
F9ULT0	Ribonucleoside-diphosphate reductase	133.23	54.37	35	35	47	82.1
F9UUC0	Endopeptidase PepO	129.96	51.10	29	29	46	71.9
F9ULZ0	Phosphoglucomutase	128.67	56.52	30	30	42	63.5
Q88XY8	Elongation factor G	128.37	57.74	32	32	45	77.0
Q88XQ0	DNA ligase	116.09	45.21	25	25	37	74.5
Q88VE1	Trigger factor	103.79	49.55	22	22	37	49.4
F9UPL0	30S ribosomal protein S1	101.00	60.84	24	24	35	47.1
F9UQ86	Translation elongation factor, GTPase,TypA/BipA family	99.52	47.39	22	22	36	68.4
Q88YH5	Phosphoglycerate kinase	94.70	66.25	24	24	36	42.8
F9UM10	Glyceraldehyde 3-phosphate dehydrogenase	93.24	52.06	16	16	25	36.4
Q88VK1	Proline--tRNA ligase	90.04	49.91	24	24	34	63.5
Q88YE7	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	82.80	49.92	21	21	27	65.4
Q88VS3	Glycine--tRNA ligase beta subunit	81.96	45.24	31	31	35	78.5
Q88YH3	Enolase 1	79.97	41.40	13	13	25	48.0
F9UN60	Phosphoenolpyruvate-protein phosphotransferase	79.64	43.06	21	21	30	63.1
F9UU91	Cell surface protein, CscB family	73.93	17.78	14	14	24	118.7
F9UPH6	DNA topoisomerase 4 subunit B	70.97	39.07	23	23	30	74.5
F9UPK1	ABC transporter, ATP-binding protein, ChvD family	69.05	44.65	20	20	25	71.3
Q88VQ8	Aspartate--tRNA ligase	68.88	38.96	22	22	28	67.6
Q88VJ4	30S ribosomal protein S2	66.92	57.30	14	14	21	30.2
F9UMH1	Asparagine synthase (Glutamine-hydrolysing)	63.09	41.55	21	21	26	73.1
Q88U67	Probable phosphoketolase 1	61.71	26.78	17	17	22	88.7
F9UN58	ATP-dependent Clp protease, ATP-binding subunit ClpE	61.66	33.96	20	20	24	81.7
Q88VJ2	D-lactate dehydrogenase	60.24	48.80	16	16	21	37.2
F9UM05	Oligopeptide ABC transporter, substrate binding protein	59.68	36.00	17	18	21	61.0

F9UNS5	6-phosphogluconate dehydrogenase, decarboxylating	56.79	43.31	17	17	20	52.9
Q88Z76	CTP synthase	56.64	36.13	17	17	21	59.7
F9US37	DNA gyrase subunit B	54.83	38.73	20	20	24	72.1
Q88VN0	Elongation factor 4 1	54.24	27.33	14	14	18	68.3
Q88YI8	UvrABC system protein B	49.99	30.13	19	19	22	76.1
F9UTT2	Fructose-bisphosphate aldolase	47.78	45.99	10	10	14	30.9
Q88Y74	GMP synthase [glutamine-hydrolyzing]	46.18	32.82	15	15	17	57.4
Q88X53	Arginine--tRNA ligase	46.01	36.48	17	17	20	62.9
Q88RX6	tRNA uridine 5-carboxymethylaminomethyl modification enzyme MnmG	44.97	31.45	18	18	20	70.7
Q88XF3	Peptide chain release factor 3	43.11	25.14	11	11	15	59.5
Q88UU1	ATP synthase subunit alpha	42.42	27.58	12	12	16	54.5
F9UNU7	Transpeptidase (Penicillin binding protein 2B)	42.38	30.09	17	17	18	72.6
P56512	L-lactate dehydrogenase 1	41.00	38.44	13	13	15	34.2
F9UL40	Transcription accessory protein, contains S1 RNA binding domain	39.99	32.92	21	21	25	80.0
Q88VJ5	Elongation factor Ts	39.31	51.37	14	14	14	31.6
Q88YY0	Glutamate--tRNA ligase	39.19	26.61	13	13	14	56.9
Q88UI4	Glucose-6-phosphate isomerase	37.31	37.56	13	13	14	49.8
Q88YW8	50S ribosomal protein L10	36.95	46.71	5	5	11	17.9
Q88XZ2	DNA-directed RNA polymerase subunit beta'	35.09	12.94	13	13	14	135.2
F9UN67	Membrane sulfatase, alkaline phosphatase superfamily protein	34.69	23.25	16	16	17	79.0
Q88XP6	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	33.52	22.36	8	8	11	53.2
Q88UU3	ATP synthase subunit beta	33.31	26.34	9	9	10	50.8
F9ULY4	UTP--glucose-1-phosphate uridylyltransferase	33.26	50.00	10	10	11	34.2
F9US18	Anaerobic ribonucleoside-triphosphate reductase	33.12	24.32	15	15	15	83.7
F9UQE9	Oligoendopeptidase F	32.96	21.06	9	9	13	67.7
Q88XY3	50S ribosomal protein L2	32.00	58.78	13	13	17	30.2
F9USW0	Linoleic acid isomerase	31.56	24.65	11	11	12	64.2
F9UNI1	GTP cyclohydrolase-2	30.80	23.02	9	9	11	43.6
F9UTA3	GMP reductase	30.60	27.42	8	8	10	39.9
Q88Z28	Lysine--tRNA ligase	30.35	26.85	13	13	14	57.6
Q88WA8	Asparagine--tRNA ligase 2	30.17	33.33	11	11	11	49.8
Q88YH4	Triosephosphate isomerase	28.88	37.70	8	8	9	27.0
F9URG7	Glucose-6-phosphate 1-dehydrogenase	28.83	25.71	9	9	11	56.8
Q6LWD9	Nickase	28.82	18.37	10	10	10	80.0
Q88V10	Alanine--tRNA ligase	28.56	13.86	10	10	11	97.2
F9UP00	ATP-dependent DNA helicase RecG	28.21	17.97	9	9	9	74.2
Q88YD2	Polyphosphate kinase	28.18	19.22	14	14	14	82.2
F9UNV9	Glutamine synthetase	27.97	35.04	12	12	13	50.9
F9UPK7	DNA-binding protein	27.97	74.73	5	5	8	9.6
F9UQB9	Cell division initiation protein DivIVA	27.88	23.28	5	5	8	26.2
Q88XX4	50S ribosomal protein L5	27.74	58.89	9	9	12	20.2
Q88VY1	ATP-dependent 6-phosphofructokinase	27.58	22.81	7	7	9	34.2
Q88V80	UDP-N-acetylmuramoylalanine--D-glutamate ligase	27.43	30.50	10	10	10	50.1
Q88YE8	Phosphoglucosamine mutase	27.31	29.71	12	12	13	48.3
F9UP45	Enoyl-[acyl-carrier-protein] reductase [NADH]	26.08	48.02	10	10	12	26.9
Q88UU0	ATP synthase subunit delta	25.80	49.17	7	7	8	20.0

Q88YL7	Protein translocase subunit SecA	25.78	14.23	9	9	12	89.5
F9UNC8	Excinuclease ABC, subunit A	25.60	14.55	8	8	8	82.3
F9UQZ0	Prophage P2a protein 6 endonuclease	24.53	24.65	7	7	8	40.6
F9UUA0	Extracellular protein,gamma-D-glutamate-meso-diaminopimelate muropeptidase	24.09	17.03	4	4	6	36.8
Q88XA9	Leucine--tRNA ligase	23.31	11.39	8	8	9	92.6
Q88YW9	50S ribosomal protein L1	23.31	41.92	10	10	12	24.8
F9UT87	Cyclopropane-fatty-acyl-phospholipid synthase	22.83	30.23	9	9	10	45.3
F9UT33	Transcription regulator, BglG family, mannitol operon	22.60	20.12	12	12	12	78.0
F9UMB1	DNA helicase	22.36	13.54	8	8	9	87.9
Q88UV8	D-alanine--D-alanine ligase	22.36	24.86	8	8	8	41.3
Q88W32	Probable manganese-dependent inorganic pyrophosphatase	21.75	33.98	9	9	10	33.6
Q88XZ3	DNA-directed RNA polymerase subunit beta	21.59	6.99	7	7	8	134.4
Q88T35	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2	21.57	35.65	7	7	9	26.1
Q88XY0	30S ribosomal protein S3	21.38	31.80	7	7	10	24.2
Q88UX2	Septation ring formation regulator EzrA	21.31	20.77	11	11	11	64.5
F9UL45	PTS system, mannose-specific EIIAB component	20.69	23.77	7	7	8	35.3
F9UKZ6	DEAD-box ATP-dependent RNA helicase CshA	20.47	16.48	7	7	7	59.0
Q88VQ7	Histidine--tRNA ligase	20.47	28.40	9	9	9	47.9
Q88XY9	30S ribosomal protein S7	20.23	43.59	5	5	6	17.8
Q88V89	Isoleucine--tRNA ligase	19.94	9.12	7	7	7	106.3
F9UNV2	Bifunctional protein: transcription regulator sugar kinase, ROK family	19.30	22.19	5	5	5	33.7
F9UQ67	Ribonuclease J	19.27	14.11	6	6	6	63.9
F9UR64	NADH peroxidase	19.17	20.84	8	8	9	48.2
F9UL91	Prophage P1 protein 5, superinfection exclusion (Cell surface N-anchored)	18.98	20.69	7	7	9	37.9
F9USZ3	Maltose phosphorylase	18.75	14.02	10	11	11	86.5
F9UP85	Maltose phosphorylase	18.71	10.96	7	8	9	85.6
Q88UZ8	DNA mismatch repair protein MutL	18.55	14.01	6	6	6	74.1
F9UMJ0	Acetolactate synthase	18.37	18.39	8	8	9	61.2
F9UTR7	NADH dehydrogenase	17.61	27.75	7	7	8	43.8
F9UTJ5	Formate C-acetyltransferase	17.55	12.23	9	9	9	84.4
Q88UX7	Valine--tRNA ligase	17.37	6.30	5	5	6	101.5
F9UQC3	Cell division protein FtsZ	17.06	18.74	6	6	6	45.0
F9UP42	Acetyl-CoA carboxylase, biotin carboxylase subunit	16.85	19.05	7	7	7	50.7
F9UMJ6	ATP-dependent Clp protease, ATP-binding subunit ClpC	16.74	10.75	6	7	7	92.6
F9US12	Cell surface protein, LPXTG-motif cell wall anchor	16.40	10.20	5	5	6	89.5
F9UM84	Glutamine ABC transporter, ATP-binding protein	16.40	27.24	5	5	6	27.4
Q88XW9	30S ribosomal protein S5	16.40	41.57	6	6	6	17.3
F9UMC8	Alkaline shock protein	15.98	38.57	4	4	5	15.8
F9UPV0	GTP pyrophosphokinase / pyrophosphohydrolase	15.93	8.48	5	5	6	86.2
Q88XW7	50S ribosomal protein L15	15.91	41.96	5	5	5	15.3
Q88W26	ATP-dependent protease ATPase subunit HslU	15.87	13.35	5	5	5	52.6
F9UQ00	Transcription termination/antitermination protein NusA	15.86	16.05	5	5	5	44.3

Q88XP7	Glutamyl-tRNA(Gln) amidotransferase subunit A	15.76	13.35	5	5	5	52.1
F9USY7	Maltodextrin-binding protein	15.60	13.19	5	5	5	45.6
Q88XB8	S-adenosylmethionine synthase	15.28	18.23	6	6	6	42.7
Q88YW7	50S ribosomal protein L7/L12	15.26	46.72	5	5	5	12.6
F9UL68	Aminopeptidase C	15.16	17.16	7	7	7	50.2
F9URD9	Cell wall hydrolase/muramidase	14.73	10.83	4	4	4	82.1
Q88XZ7	Serine--tRNA ligase 2	14.62	14.12	5	5	5	48.1
F9UR85	Aspartate-semialdehyde dehydrogenase	14.57	11.61	3	3	4	38.2
Q88TV5	Tyrosine--tRNA ligase	14.47	16.03	6	6	6	47.0
F9UMC9	Alkaline shock protein	14.41	38.36	3	3	5	16.1
Q88XY5	50S ribosomal protein L4	14.11	26.57	4	4	4	22.6
F9UPS8	DNA primase	13.91	11.82	6	6	6	70.4
F9UPA5	Transpeptidase-transglycosylase (Penicillin binding protein 1A)	13.83	8.60	5	5	5	83.1
F9URQ9	Uncharacterized protein	13.78	9.21	6	6	6	76.2
F9UP39	3-oxoacyl-[acyl-carrier-protein] synthase 2	13.74	10.24	3	3	4	42.4
F9UPH5	DNA topoisomerase 4 subunit A	13.73	4.66	3	3	4	91.6
F9UNU0	Endolytic murein transglycosylase	13.64	15.21	5	5	5	44.7
Q88VE2	ATP-dependent Clp protease ATP-binding subunit ClpX	13.46	10.21	3	3	4	46.5
F9UQH9	Extracellular protein, DUF336 family	13.43	28.82	4	4	4	18.0
Q88YC0	Xaa-Pro dipeptidyl-peptidase	13.31	5.54	3	3	4	91.5
F9UMU5	ATP-dependent DNA helicase	13.19	8.08	5	5	6	85.0
F9UT53	Cystathionine beta-lyase / cystathionine gamma-lyase	12.87	16.54	5	5	5	40.8
Q88XY6	50S ribosomal protein L3	12.75	28.71	4	4	4	22.7
F9UN93	Xaa-His dipeptidase	12.74	13.49	5	5	5	50.8
F9UTA5	Ribosome-binding ATPase YchF	12.27	20.77	6	6	6	39.9
P71479	Bifunctional protein PyrR 1	12.06	30.00	5	5	5	19.8
F9UTT6	Dipeptidase	11.88	9.15	3	3	4	52.9
Q88Z84	Ribose-phosphate pyrophosphokinase 1	11.74	21.17	7	7	7	35.9
F9URU9	Extracellular transglycosylase, with LysM peptidoglycan binding domain	11.64	13.56	4	4	5	35.0
Q88VK7	Translation initiation factor IF-2	11.59	9.21	7	7	7	93.6
Q88UZ4	Protein RecA	11.45	16.58	5	5	5	40.6
Q88WG0	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta 2	11.20	16.37	3	3	3	30.9
F9UPS7	RNA polymerase sigma factor SigA	11.19	11.96	4	4	4	41.4
Q88XW0	DNA-directed RNA polymerase subunit alpha	10.98	12.10	4	4	4	34.8
F9UMS0	Citrate lyase alpha chain	10.60	13.09	5	5	5	54.9
F9UL89	Prophage P1 protein 2, mitogenic factor, cell surface lipoprotein	10.59	9.30	3	3	4	41.8
F9UQC4	Cell division protein FtsA	10.47	17.41	6	6	6	48.3
F9UQQ1	L-2-hydroxyisocaproate dehydrogenase	10.44	16.07	4	4	4	32.5
Q88VM6	D-alanine--D-alanyl carrier protein ligase	10.28	17.52	7	7	8	56.0
F9UTG0	Glutathione reductase	10.27	10.81	4	4	4	48.2
Q88XU8	50S ribosomal protein L13	10.09	31.97	4	4	4	16.2
F9ULV5	ABC transporter, ATP-binding protein	9.54	7.58	4	4	4	74.1
F9UUE9	Beta-galactosidase, large subunit	9.40	6.87	4	4	4	72.1
Q88Y23	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase	9.25	8.72	3	3	3	53.8
F9UPH4	Transcription regulator, LysR family	9.25	12.54	4	4	4	36.8

F9UNZ3	Serine/threonine protein kinase	9.08	3.71	2	2	3	74.5
P77886	Carbamoyl-phosphate synthase pyrimidine-specific large chain	8.89	4.82	5	5	6	115.7
F9UNG4	Transpeptidase-transglycosylase (Penicillin binding protein 2A)	8.78	5.78	3	3	3	77.7
F9UMD4	Aminopeptidase	8.76	5.57	4	4	4	93.9
F9UL42	Homoserine dehydrogenase	8.60	8.88	3	3	3	46.6
Q88VM1	Chaperone protein DnaJ	8.59	11.05	3	3	4	40.5
F9UT98	Serine-type D-Ala-D-Ala carboxypeptidase	8.55	8.78	3	3	3	46.9
F9UM27	Phosphate acetyltransferase	8.50	15.38	4	4	4	34.4
Q88XX1	50S ribosomal protein L6	8.48	23.60	4	4	4	19.3
F9UPI3	DNA topoisomerase 1	8.47	5.73	4	4	4	80.7
Q88WJ5	30S ribosomal protein S16	8.44	43.33	2	2	2	10.2
F9UMV3	Transcription regulator, TetR family	8.30	21.99	4	4	4	21.6
F9US66	Serine protease HtrA	8.30	13.33	4	4	4	43.1
F9UMX4	UDP-galactopyranose mutase	8.29	14.75	6	6	6	43.2
F9UU18	DNA helicase	8.28	6.37	4	4	4	87.8
F9UL47	PTS system, mannose-specific EIID component	8.05	11.48	3	3	3	33.6
Q88WU6	50S ribosomal protein L20	8.01	19.49	3	3	3	13.6
Q88X05	Foldase protein PrsA 1	8.00	10.07	3	3	3	32.6
Q88Z31	ATP-dependent zinc metalloprotease FtsH	7.75	4.97	3	3	3	80.8
F9UNW0	Uncharacterized protein	7.57	12.54	3	3	3	34.5
F9UL33	N-acetylglucosamine-6-phosphate deacetylase	7.35	9.79	3	3	3	41.4
F9ULW6	Ribosome hibernation promoting factor	7.34	24.47	4	4	5	21.7
P77883	Aspartate carbamoyltransferase	7.33	16.40	3	3	3	34.7
Q88VZ6	GTPase Der	7.30	9.17	4	4	4	48.7
F9UN51	Oligopeptide ABC transporter, substrate binding protein	7.18	7.50	3	4	4	60.3
F9UQS7	UDP-N-acetylmuramyl tripeptide synthase	7.06	7.37	3	3	4	49.7
Q88VX7	Chaperone protein ClpB	7.05	5.42	3	4	4	96.5
F9ULT4	DNA polymerase III subunit gamma/tau	7.03	7.26	3	3	4	62.7
F9UPM8	Peptidase T	7.02	5.83	2	2	3	45.1
Q88V23	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferas	6.96	12.71	2	2	2	24.5
F9UP44	Acetyl-CoA carboxylase, carboxyl transferase subunit alpha	6.96	20.31	3	3	3	27.6
F9UQH6	Catabolite control protein A	6.84	12.20	4	4	5	36.3
F9UP94	Aminotransferase	6.82	7.73	3	3	4	43.8
F9URZ3	Hypothetical membrane protein	6.73	4.16	4	4	4	115.2
Q88UW5	Glutathione biosynthesis bifunctional protein GshAB	6.68	4.39	3	3	3	83.0
F9UMN8	Lipoprotein, FMN-binding protein	6.65	13.64	3	3	3	32.9
F9UNY1	Glycine betaine/carnitine/choline ABC transporter, ATP-binding protein	6.51	8.04	3	3	3	44.4
Q88XY4	50S ribosomal protein L23	6.48	29.90	2	2	2	11.1
Q88XX0	50S ribosomal protein L18	6.44	22.88	3	3	3	13.0
Q88WR2	Phenylalanine--tRNA ligase beta subunit	6.36	7.45	4	4	4	88.1
Q88WN5	50S ribosomal protein L21	6.30	31.37	2	2	2	11.0
F9UQM2	Site-determining protein	6.25	10.07	2	2	2	29.1
F9UN64	Serine-type D-Ala-D-Ala carboxypeptidase	6.24	6.07	2	2	2	45.2
F9UN65	Aminotransferase	6.17	5.57	2	2	2	43.8

Q88UX0	30S ribosomal protein S4	6.17	16.83	4	4	4	22.9
Q88VR5	30S ribosomal protein S21	6.04	43.55	4	4	4	7.6
F9UP07	Signal recognition particle protein	6.02	4.75	2	2	2	53.9
Q88WZ5	UDP-N-acetylmuramate--L-alanine ligase	5.91	11.01	4	4	4	48.7
F9UMN7	NADH dehydrogenase, membrane-anchored	5.87	9.75	6	6	6	71.9
F9UQM6	Cell shape determining protein MreB	5.64	10.48	4	4	5	35.1
F9ULY7	Thioredoxin reductase	5.64	12.82	4	4	4	33.4
Q06115	Choloylglycine hydrolase	5.47	8.64	3	3	3	37.0
Q88XW2	30S ribosomal protein S13	5.45	17.36	2	2	2	13.6
F9UQA0	Extracellular protein, NlpC/P60 family,gamma-D-glutamate-meso-diaminopimelate muropeptidase	5.36	10.28	3	3	3	48.3
Q88V33	Probable transcriptional regulatory protein lp_2253	5.22	12.40	4	4	4	26.4
F9URK6	NADPH-dependent FMN reductase family protein	5.15	7.89	3	3	3	47.4
Q88UT5	Serine hydroxymethyltransferase	5.07	7.77	3	3	3	44.4
F9USR0	Elongation factor G	5.07	3.42	2	2	2	73.9
F9UQ80	Pyruvate carboxylase	5.07	5.16	5	5	5	127.1
Q88YI7	UvrABC system protein A	4.74	3.79	3	3	3	104.9
F9UU82	2,5 diketo-D-gluconic acid-like reductase, NADP dependent (Promiscuous)	4.70	12.59	3	3	3	31.7
Q88XU7	30S ribosomal protein S9	4.60	16.92	2	2	2	14.6
F9UP38	3-oxoacyl-[acyl-carrier protein] reductase	4.45	10.74	2	2	2	25.7
F9UQ85	Extracellular protein, cell-wall anchored	4.38	5.08	2	2	2	45.8
Q88XY1	50S ribosomal protein L22	4.32	14.78	2	2	2	12.5
F9UP59	Cyclopropane-fatty-acyl-phospholipid synthase	4.31	5.38	2	2	2	44.7
Q88UT2	Peptide chain release factor 1	4.14	5.28	2	2	2	41.0
Q9RE01	Uracil phosphoribosyltransferase	4.05	7.66	2	2	2	23.0
F9UT64	Glycerol-3-phosphate cytidylyltransferase	3.99	11.94	2	2	2	15.8
F9UM19	Ribonuclease R	3.98	3.21	2	2	2	92.3
Q88WG9	3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ	3.38	14.97	2	2	2	16.0
F9UMY7	dTDP-glucose 4,6-dehydratase	3.10	6.14	2	2	3	38.6
F9UT61	PTS system, trehalose-specific IIBC component	2.91	3.16	2	2	2	69.4
F9UP37	Malonyl CoA-acyl carrier protein transacylase	2.87	8.06	2	2	3	33.3
Q88UZ7	DNA mismatch repair protein MutS	2.66	2.68	2	2	2	99.9
F9URP6	6-phospho-beta-glucosidase	2.64	10.00	4	4	4	54.7
Q88UT9	ATP synthase subunit b	2.58	12.28	2	2	2	18.7
F9UTV3	Multicopper oxidase	2.53	5.99	3	3	3	56.8
Q88WU8	Translation initiation factor IF-3	2.07	13.29	2	2	2	19.6
F9ULD3	Catalase	2.03	3.51	2	2	2	55.3
F9UMI1	Metal-dependent regulator	1.89	7.44	2	2	2	23.9
F9ULI3	Alpha-1,2-mannosidase	1.85	2.80	2	2	2	80.8
F9UQS9	Mannose-6-phosphate isomerase	1.80	5.92	2	2	2	35.9
Q88YI4	Nucleotide-binding protein lp_0779	1.72	7.48	2	2	2	33.3
F9US34	Beta sliding clamp	0.00	9.50	4	4	4	41.5

Band # 6

Accession	Description	Score	Coverage	# Unique Peptides	# Peptides	# PSMs	MW [kDa]
F9UM10	Glyceraldehyde 3-phosphate dehydrogenase	311.72	69.71	21	21	108	36.4
Q88VJ4	30S ribosomal protein S2	258.60	70.79	22	22	96	30.2
P56512	L-lactate dehydrogenase 1	169.73	49.06	22	22	66	34.2
Q88VJ2	D-lactate dehydrogenase	131.32	58.73	22	22	54	37.2
Q88VJ5	Elongation factor Ts	99.04	55.14	17	17	30	31.6
F9UL45	PTS system, mannose-specific EIIAB component	95.61	51.23	16	16	34	35.3
Q88Z84	Ribose-phosphate pyrophosphokinase 1	91.06	41.72	15	15	28	35.9
Q88Z14	NH(3)-dependent NAD(+) synthetase	76.34	53.45	13	13	25	30.6
F9UTA7	Chomosome partitioning protein, DNA-binding protein	66.02	53.04	17	17	25	33.1
F9ULU4	UDP-glucose 4-epimerase	65.50	50.15	11	12	21	36.1
Q88VE0	Elongation factor Tu	59.26	51.14	15	15	22	43.4
F9UU82	2,5 diketo-D-gluconic acid-like reductase, NADP dependent (Promiscuous)	58.21	47.90	13	13	20	31.7
F9UPH4	Transcription regulator, LysR family	57.63	52.98	13	13	18	36.8
F9UM27	Phosphate acetyltransferase	57.06	47.38	11	11	19	34.4
Q88VA5	Putative ribose-phosphate pyrophosphokinase 2	56.90	41.12	11	11	19	35.0
F9UTA3	GMP reductase	54.90	37.08	11	11	20	39.9
F9UPM3	Pyruvate kinase	54.86	31.23	14	14	17	62.8
F9ULY4	UTP--glucose-1-phosphate uridylyltransferase	52.94	39.22	12	12	18	34.2
F9UMR9	Citrate lyase, beta chain	52.61	51.97	13	13	20	33.2
F9UUE7	UDP-glucose 4-epimerase	48.36	45.21	12	13	17	36.4
F9UPY0	Serine-type D-Ala-D-Ala carboxypeptidase	48.13	40.15	13	13	17	43.3
Q88VS2	Glycine--tRNA ligase alpha subunit	47.37	35.12	10	10	19	34.5
F9UT87	Cyclopropane-fatty-acyl-phospholipid synthase	47.36	29.22	9	9	14	45.3
F9UTT2	Fructose-bisphosphate aldolase	47.04	45.99	10	10	15	30.9
F9UQB9	Cell division initiation protein DivIVA	46.00	35.34	9	9	16	26.2
F9ULY7	Thioredoxin reductase	45.98	50.64	12	12	13	33.4
Q88VG6	Ribonuclease Z	45.81	55.31	15	15	19	34.2
Q88VY1	ATP-dependent 6-phosphofructokinase	45.62	31.25	9	9	14	34.2
F9UMI5	Transcriptional attenuator, cell envelope-related, LytR family	44.72	41.45	11	11	14	37.7
F9UQ92	Pyruvate dehydrogenase complex, E1 component,beta subunit	43.95	44.62	13	13	16	35.4
Q88YI2	Probable cell division protein WhiA	42.27	39.35	12	12	14	35.1
F9UQ43	Tagatose-6-phosphate kinase	41.60	39.02	9	9	12	32.4
Q88YK1	Glycerol-3-phosphate dehydrogenase [NAD(P)+]	41.40	28.11	6	6	10	36.6
F9USL4	Aldo/keto reductase family protein	40.87	36.59	11	11	15	35.5
F9UQH2	Ribokinase	40.25	32.03	9	9	12	31.7
F9UMT8	Heptaprenyl diphosphate synthase component II	39.95	31.08	8	8	12	35.9
F9UU48	Penicillin V acylase family protein	39.54	32.62	6	6	11	36.1
P77883	Aspartate carbamoyltransferase	39.41	33.76	8	8	13	34.7
F9UQQ9	Cell shape determining protein MreB	39.04	28.83	8	8	13	35.8
Q88W05	Thymidylate synthase	38.79	38.61	12	12	15	36.0
F9UQM6	Cell shape determining protein MreB	38.72	37.72	11	11	14	35.1

F9UTW5	Glycine betaine/carnitine/choline ABC transporter, ATP-binding protein	38.29	43.96	12	12	14	35.8
Q88WU9	Threonine--tRNA ligase	36.63	24.92	12	12	13	73.8
Q88XY3	50S ribosomal protein L2	35.92	59.86	13	13	18	30.2
Q88WL3	Methionyl-tRNA formyltransferase	35.72	34.38	10	10	13	34.4
F9UMC8	Alkaline shock protein	35.15	63.57	9	9	12	15.8
F9URD8	Lipoate--protein ligase	35.13	32.64	10	10	13	38.2
F9UUF4	Transcription regulator. LacI family, raffinose related	33.79	32.02	11	11	13	37.1
F9UQE3	6-phosphogluconolactonase	33.68	33.92	9	9	13	36.5
F9UQ89	Malate/L-lactate/L-2-hydroxyisocapronate dehydrogenase	33.66	34.52	9	9	11	33.1
Q88VL8	Heat-inducible transcription repressor HrcA	33.44	41.50	14	14	17	38.9
F9UU33	Uncharacterized protein	33.24	25.39	7	7	11	36.4
Q88UU2	ATP synthase gamma chain	32.57	30.57	10	10	13	34.5
F9UPM9	GTP cyclohydrolase 1 type 2 homolog	32.17	21.47	7	7	11	37.2
F9UNX0	Xaa-Pro aminopeptidase	31.50	25.50	6	6	9	37.8
F9URL2	L-asparaginase	31.30	32.41	7	7	11	34.4
F9UMR5	Citrate lyase regulator, SorC family	30.64	28.44	8	8	10	35.6
F9UT53	Cystathionine beta-lyase / cystathionine gamma-lyase	30.11	24.15	8	8	13	40.8
F9UTJ4	Beta-lactamase family protein	30.07	24.62	7	7	13	37.3
Q88YH4	Triosephosphate isomerase	29.76	40.08	9	9	11	27.0
Q88VQ7	Histidine--tRNA ligase	29.32	26.76	10	10	14	47.9
F9UQJ8	Phosphoesterase, DHH family	29.22	28.62	7	7	9	34.5
Q88XW0	DNA-directed RNA polymerase subunit alpha	28.80	24.84	8	8	10	34.8
F9UPY8	Riboflavin biosynthesis protein	28.27	36.94	10	10	13	36.9
F9UMW1	Oxidoreductase, NAD-dependent	28.21	33.43	8	8	11	37.1
F9UT17	Acetate kinase	27.94	28.61	9	9	11	42.9
Q88W32	Probable manganese-dependent inorganic pyrophosphatase	27.82	32.36	10	10	11	33.6
F9ULS9	Ribonucleoside-diphosphate reductase, beta chain	27.68	20.83	9	9	12	39.1
Q88WK2	Phosphate acyltransferase	27.45	26.72	8	8	10	37.4
F9ULC7	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	27.32	27.68	10	10	11	44.4
F9UT54	Cystathionine beta-synthase	26.81	43.89	8	8	10	32.2
Q88YN0	tRNA N6-adenosine threonylcarbamoyltransferase	26.66	25.00	6	6	10	37.5
F9UM03	Putative gluconeogenesis factor	25.89	25.53	6	6	7	36.6
F9UL89	Prophage P1 protein 2, mitogenic factor, cell surface lipoprotein	25.73	32.30	11	11	12	41.8
F9UMN8	Lipoprotein, FMN-binding protein	25.67	19.48	5	5	8	32.9
F9UMV7	Transcription regulator, RpiR family	24.75	40.41	9	9	10	31.7
Q88WG8	3-oxoacyl-[acyl-carrier-protein] synthase 3 protein 2	24.54	21.65	6	6	12	35.3
Q88V90	Diaminopimelate epimerase	24.52	24.70	7	7	8	35.9
Q88VP9	Ribosomal protein L11 methyltransferase	24.48	28.16	6	6	9	34.1
F9US66	Serine protease HtrA	24.25	26.67	8	8	8	43.1
F9UUA8	Peroxidase	24.23	31.55	8	8	9	35.9
F9UNV2	Bifunctional protein: transcription regulator sugar kinase, ROK family	24.03	24.38	6	6	7	33.7
F9UMD1	Extracellular lipoprotein	23.92	26.49	7	7	9	36.8
F9UQP9	Zinc-type alcohol dehydrogenase-like protein	23.77	21.35	6	6	9	36.1
Q88T35	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2	23.60	37.83	8	8	12	26.1

F9UQN3	Cysteine desulfurase	23.27	18.75	6	6	8	42.3
Q88XY0	30S ribosomal protein S3	23.10	43.78	10	10	12	24.2
F9URN7	Uncharacterized protein	23.03	23.69	9	9	11	40.7
Q88XX4	50S ribosomal protein L5	22.99	39.44	6	6	9	20.2
Q88YH7	Central glycolytic genes regulator	22.70	24.78	10	10	10	38.1
F9URT3	Purine nucleosidase, inosine-uridine preferring	22.64	25.46	6	6	7	35.4
F9URJ0	Uncharacterized protein	22.17	30.33	7	7	7	32.9
F9UQS9	Mannose-6-phosphate isomerase	22.16	27.73	7	7	8	35.9
Q88UH9	UPF0210 protein lp_2507	21.27	14.09	6	6	7	46.6
F9UQL5	Transcription regulator, Xre family	21.23	27.59	7	7	8	31.3
Q88YH3	Enolase 1	21.20	18.55	7	7	8	48.0
Q88YW9	50S ribosomal protein L1	21.05	28.38	7	7	8	24.8
F9UUF5	Aldo/keto reductase family protein	20.94	20.00	5	5	8	37.9
F9ULZ0	Phosphoglucomutase	20.93	16.00	7	7	8	63.5
F9URK8	Lipoate--protein ligase	20.82	25.00	8	8	10	37.2
Q06115	Choloylglycine hydrolase	20.51	26.85	8	8	11	37.0
F9UQH7	Xaa-Pro dipeptidase	19.75	17.07	6	6	9	41.1
Q88WK9	Small ribosomal subunit biogenesis GTPase RsgA 1	19.71	25.17	6	6	7	32.9
F9ULK7	Ribokinase	19.00	26.47	7	7	9	32.0
Q88V81	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	18.79	21.76	7	7	7	38.7
O08445	Alanine racemase	18.64	17.33	5	5	7	40.7
F9UP59	Cyclopropane-fatty-acyl-phospholipid synthase	18.53	13.85	5	5	7	44.7
Q88UC0	Ferredoxin--NADP reductase	17.88	19.34	6	6	7	36.1
Q88X05	Foldase protein PrsA 1	17.87	28.19	7	7	7	32.6
F9UQS3	Threonylcarbamoyl-AMP synthase	17.86	25.66	7	7	7	36.3
F9UMU0	Oxidoreductase, NAD(P)-dependent	17.67	21.91	6	6	8	35.9
F9UMA6	Phosphohydrolase, MutT/nudix family	17.55	22.69	7	7	8	40.0
F9UN62	Glucosyl-diacylglycerol 6-beta-glucosyltransferase	17.35	26.47	8	8	8	39.6
Q88UU0	ATP synthase subunit delta	17.05	31.49	5	5	6	20.0
Q88YH5	Phosphoglycerate kinase	17.00	19.75	9	9	11	42.8
F9UU28	tRNA-dihydrouridine synthase	16.97	18.69	5	5	7	37.1
Q88WA8	Asparagine--tRNA ligase 2	16.83	14.81	6	6	6	49.8
F9UQZ0	Prophage P2a protein 6 endonuclease	16.83	24.65	7	7	7	40.6
Q88Y75	Pantothenate kinase	16.71	21.36	6	6	6	36.5
Q88UX0	30S ribosomal protein S4	16.65	18.32	4	4	7	22.9
F9URD4	Extracellular protein	16.65	15.91	3	3	5	23.1
F9UPH8	Aldose 1-epimerase family protein	16.56	16.10	5	5	7	32.7
Q88WB6	Isopentenyl-diphosphate delta-isomerase	16.34	20.40	5	5	5	38.0
F9UMY7	dTDP-glucose 4,6-dehydratase	15.91	13.45	4	4	6	38.6
F9UPL0	30S ribosomal protein S1	15.90	21.91	9	9	9	47.1
F9UMU4	Formate-dependent phosphoribosylglycinamide formyltransferase	15.67	7.97	2	2	4	42.8
Q88XW9	30S ribosomal protein S5	15.35	40.36	7	7	7	17.3
F9USN8	DNA-binding ferritin-like protein, DPS family	15.23	43.23	6	6	7	18.0
Q88XU8	50S ribosomal protein L13	15.11	30.61	3	3	4	16.2
Q88WG0	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta 2	14.92	23.13	5	5	6	30.9

F9UM44	Maltose epimerase	14.81	16.05	6	6	7	38.9
Q88V76	Ribosomal RNA small subunit methyltransferase H	14.69	15.67	5	5	5	35.8
F9UST5	Medium chain dehydrogenases/reductase (MDR)/zinc-dependent alcohol dehydrogenase-like family protein	14.65	11.69	3	3	5	34.1
F9UN64	Serine-type D-Ala-D-Ala carboxypeptidase	14.44	12.38	4	4	5	45.2
Q88V04	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	14.25	21.51	6	6	7	39.0
F9UNC5	Transcription regulator, MarR family	14.16	26.42	3	3	7	18.2
F9URA1	Ribonuclease H	14.07	23.15	7	7	7	33.0
P71479	Bifunctional protein PyrR 1	13.90	30.00	5	5	6	19.8
Q88YK3	HPr kinase/phosphorylase	13.75	19.05	6	6	7	34.8
F9ULE0	NAD-independent L-lactate dehydrogenase	13.74	13.66	4	4	4	38.7
F9UU20	Tryptophan-tRNA synthetase	13.68	23.08	6	6	7	37.6
F9UUB1	Endoglucanase E-like protein, SGNH hydrolase family	13.68	18.58	5	5	5	35.8
Q88VM0	Chaperone protein DnaK	13.59	9.16	5	5	5	66.7
F9UPJ2	Flavin monooxygenase, luciferase-like monooxygenase family	13.44	18.47	5	5	5	38.8
F9UP45	Enoyl-[acyl-carrier-protein] reductase [NADH]	13.43	36.90	8	8	9	26.9
F9UR55	2-dehydropantoate 2-reductase	13.36	14.81	4	4	6	36.9
F9US21	FAD:protein FMN transferase	13.20	15.32	4	4	4	36.6
F9UTH2	Methonine synthase (Cobalamine-independent),C-terminal domain	13.11	18.01	6	6	8	42.0
Q88YW7	50S ribosomal protein L7/L12	12.83	44.26	5	5	5	12.6
F9UU93	Cell surface protein, CscB family	12.63	13.75	3	3	4	27.4
P59390	L-lactate dehydrogenase 2	12.34	17.48	6	6	6	33.1
F9UNW0	Uncharacterized protein	12.19	27.12	8	8	9	34.5
F9UMV3	Transcription regulator, TetR family	12.17	26.18	5	5	5	21.6
F9USY7	Maltodextrin-binding protein	12.12	12.95	4	4	4	45.6
F9UMX5	Polysaccharide biosynthesis protein	12.05	13.64	3	3	4	38.5
F9USY4	Transcription regulator, LacI family,maltose-related	11.74	21.24	5	5	5	37.9
F9UP94	Aminotransferase	11.74	11.47	3	3	4	43.8
Q88VS0	GTPase Era	11.71	26.16	7	7	7	34.3
Q88W26	ATP-dependent protease ATPase subunit HslU	11.70	5.93	2	2	3	52.6
F9URB2	NAD(P)-dependent oxidoreductase	11.66	20.62	6	6	6	35.6
F9ULD9	ATP-dependent Clp protease, ATP-binding subunit ClpL	11.63	9.52	5	5	5	77.7
Q88XY9	30S ribosomal protein S7	11.46	41.03	5	5	6	17.8
F9UPJ8	DegV family protein	11.28	13.78	3	3	4	30.1
F9UTV9	Biotin carboxyl carrier protein of acetyl-CoA carboxylase	11.27	24.21	3	3	4	20.4
F9USZ9	Oligosucrose operon repressor	11.20	14.11	4	4	4	36.2
F9UPT5	Phosphate starvation-inducible protein	11.20	12.62	3	3	4	35.8
Q88XY6	50S ribosomal protein L3	11.14	24.40	5	5	5	22.7
Q88UV2	Methionine import ATP-binding protein MetN 2	11.10	22.16	6	6	6	37.7
Q88YW8	50S ribosomal protein L10	11.07	20.96	3	3	4	17.9
F9UNI1	GTP cyclohydrolase-2	10.90	13.12	5	5	5	43.6
F9UPL2	Extracellular protein, with LysM peptidoglycan binding domain	10.84	17.62	3	3	4	21.6
F9UTF3	DegV family protein	10.82	12.19	2	2	3	29.6
F9ULI2	Transcription regulator, GntR family	10.75	12.71	4	4	4	39.4

F9UNG8	Metal-dependent phosphohydrolase, HD family	10.64	16.26	4	4	5	37.0
F9UL91	Prophage P1 protein 5, superinfection exclusion (Cell surface N-anchored)	10.55	16.38	6	6	6	37.9
Q88XZ2	DNA-directed RNA polymerase subunit beta'	10.47	4.12	4	4	5	135.2
F9US22	tRNA-dihydrouridine synthase	10.44	14.97	4	4	4	37.8
F9UT98	Serine-type D-Ala-D-Ala carboxypeptidase	10.36	6.53	2	2	3	46.9
P77884	Dihydroorotase	10.24	6.74	2	2	3	45.4
F9UQG6	Type I restriction-modification system,adenine-specific methyltransferase subunit	10.21	16.07	5	5	5	37.2
F9UM06	2-hydroxyacid dehydrogenase	10.17	10.80	3	3	4	34.8
Q88V96	tRNA-specific 2-thiouridylase MnmA	9.98	12.57	3	3	4	42.0
F9UQP6	Transcription regulator, Xre family	9.93	14.09	4	4	4	34.2
F9UN55	Oligopeptide ABC transporter, ATP-binding protein	9.63	12.46	3	3	4	36.6
F9ULH5	Transcription regulator, LacI family	9.55	15.82	4	4	4	36.6
F9UMX4	UDP-galactopyranose mutase	9.53	10.72	4	4	5	43.2
F9UMV1	Diacylglycerol kinase family protein	9.51	11.18	4	4	5	37.0
Q88YE7	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	9.43	7.93	4	4	4	65.4
F9UPK7	DNA-binding protein	9.34	31.87	2	2	3	9.6
Q88VR2	Probable endonuclease 4	9.13	14.09	3	3	3	33.0
F9UP39	3-oxoacyl-[acyl-carrier-protein] synthase 2	9.06	11.95	4	4	4	42.4
F9UQQ1	L-2-hydroxyisocaproate dehydrogenase	9.04	12.13	3	3	3	32.5
Q88YZ4	3-oxoacyl-[acyl-carrier-protein] synthase 3 protein 1	9.03	11.46	2	2	2	34.4
F9USY0	Dihydroxyacetone phosphotransferase,dihydroxyacetone binding subunit	9.00	10.54	4	4	5	36.1
F9UP89	Diphosphomevalonate decarboxylase	8.98	15.38	4	4	4	35.2
F9UMH0	Aspartokinase	8.95	17.75	5	5	5	42.8
F9UTI2	Dihydropteroate synthase	8.92	9.42	4	4	4	43.4
F9UN47	Uncharacterized protein	8.88	16.72	4	4	4	38.2
Q88Y74	GMP synthase [glutamine-hydrolyzing]	8.75	9.65	5	5	5	57.4
F9UP38	3-oxoacyl-[acyl-carrier protein] reductase	8.71	12.81	3	3	3	25.7
F9UM05	Oligopeptide ABC transporter, substrate binding protein	8.42	6.91	3	3	3	61.0
F9UP42	Acetyl-CoA carboxylase, biotin carboxylase subunit	8.35	5.63	2	2	3	50.7
F9ULW6	Ribosome hibernation promoting factor	8.33	17.55	3	3	3	21.7
F9UTQ0	Transport protein, MMPL family	8.29	3.49	3	3	3	143.2
Q88XY5	50S ribosomal protein L4	8.21	23.67	4	4	4	22.6
F9USZ2	Maltodextrin ABC transporter, ATP-binding protein	8.20	8.97	2	2	3	41.3
F9UNS5	6-phosphogluconate dehydrogenase, decarboxylating	8.18	11.09	4	4	4	52.9
Q88XV9	50S ribosomal protein L17	8.12	29.13	3	3	3	14.2
Q88XW2	30S ribosomal protein S13	8.12	23.97	3	3	3	13.6
F9URA0	Purine nucleosidase	7.95	8.81	2	2	3	34.1
Q88WU6	50S ribosomal protein L20	7.82	24.58	3	3	3	13.6
F9UTR7	NADH dehydrogenase	7.69	13.75	4	4	4	43.8
F9UL33	N-acetylglucosamine-6-phosphate deacetylase	7.37	9.52	3	3	3	41.4
F9ULU0	DNA-directed DNA polymerase III, delta' subunit	7.29	11.21	3	3	3	37.2
Q88XB8	S-adenosylmethionine synthase	7.27	9.11	3	3	3	42.7

F9UQT2	Branched-chain amino acid aminotransferase	7.12	10.82	3	3	3	37.9
Q88WN5	50S ribosomal protein L21	7.09	27.45	2	2	2	11.0
F9UM66	Proline iminopeptidase	7.06	10.26	2	2	2	34.5
F9URE6	Ribosomal large subunit pseudouridylate synthase	6.82	18.33	3	3	3	33.3
Q88YE8	Phosphoglucosamine mutase	6.74	5.99	2	2	2	48.3
Q88V23	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase	6.64	12.71	2	2	2	24.5
F9UP44	Acetyl-CoA carboxylase, carboxyl transferase subunit alpha	6.60	9.77	2	2	2	27.6
F9UM84	Glutamine ABC transporter, ATP-binding protein	6.58	10.98	2	2	2	27.4
F9UPP0	NAD(P)(H)-dependent oxidoreductase, quinone oxidoreductase (QOR) family	6.56	11.11	3	3	3	34.9
Q88W73	Carbamoyl-phosphate synthase small chain	6.53	6.11	2	2	2	39.2
Q88V33	Probable transcriptional regulatory protein lp_2253	6.44	15.29	4	4	5	26.4
Q88UU3	ATP synthase subunit beta	6.41	5.14	2	2	2	50.8
Q8GH68	Divalent metal cation transporter MntH	6.39	9.05	5	5	5	50.3
F9UTT9	Bifunctional ligase/repressor BirA	6.36	9.91	3	3	3	35.3
F9UT04	Transcription regulator, LysR family	6.31	10.20	3	3	3	34.1
Q88WZ5	UDP-N-acetylmuramate--L-alanine ligase	6.20	6.65	2	2	2	48.7
F9UPR0	Oxidoreductase, medium chain dehydrogenases/reductase (MDR)/zinc-dependent alcohol dehydrogenase-like family	6.13	10.19	2	2	2	33.6
F9UNQ0	Primosomal protein DnaI	6.07	11.90	3	3	5	35.2
F9UNG1	Aminotransferase	5.99	5.63	2	2	2	41.6
F9UP88	Phosphomevalonate kinase	5.97	7.99	3	3	3	39.5
Q88XY8	Elongation factor G	5.82	6.88	4	4	5	77.0
Q88XX2	30S ribosomal protein S8	5.68	23.66	3	3	3	14.6
Q88WR3	Phenylalanine--tRNA ligase alpha subunit	5.60	14.37	5	5	5	39.3
F9UQB3	Cysteine desulfurase	5.60	4.92	2	2	2	42.3
F9USH3	1,3-propanediol dehydrogenase	5.55	6.67	2	2	2	42.1
Q88XX1	50S ribosomal protein L6	5.53	17.42	3	3	3	19.3
Q88VJ7	Ribosome-recycling factor	5.49	15.51	2	2	2	20.6
Q88YM8	Redox-sensing transcriptional repressor Rex	5.36	9.78	2	2	2	25.2
F9URX3	Alcohol dehydrogenase, zinc-binding	5.33	6.07	2	2	2	36.7
Q88U67	Probable phosphoketolase 1	5.29	2.79	2	2	2	88.7
Q88SV5	GMP reductase	5.14	7.08	2	2	2	35.4
F9UQH9	Extracellular protein, DUF336 family	5.13	13.53	2	2	2	18.0
Q88Z93	Ribosomal RNA small subunit methyltransferase A	5.01	11.49	3	3	3	32.7
Q88VJ6	Uridylate kinase	5.00	13.33	3	3	3	25.9
F9UR85	Aspartate-semialdehyde dehydrogenase	5.00	7.08	2	2	2	38.2
F9UQS4	Release factor glutamine methyltransferase	4.99	5.90	2	2	2	31.6
F9UMH1	Asparagine synthase (Glutamine-hydrolysing)	4.99	5.21	2	2	2	73.1
Q88TV5	Tyrosine--tRNA ligase	4.99	5.74	2	2	2	47.0
F9UPK2	Uncharacterized protein	4.91	20.00	3	3	3	17.1
Q88VL9	Protein GrpE	4.89	11.56	2	2	3	21.4
F9UTM5	Small heat shock protein	4.85	22.45	2	2	2	16.7
P77885	Carbamoyl-phosphate synthase pyrimidine-specific small chain	4.82	10.16	3	3	3	40.0

Q88XY1	50S ribosomal protein L22	4.75	14.78	2	2	2	12.5
F9UMM5	Protein translocase subunit SecY	4.74	7.42	3	3	3	47.2
Q88VH0	Adenine phosphoribosyltransferase	4.70	15.70	2	2	2	18.9
Q88XX6	50S ribosomal protein L14	4.68	18.85	2	2	2	13.1
F9UL19	Hypoxanthine phosphoribosyltransferase	4.60	11.11	2	2	2	20.3
Q88UZ4	Protein RecA	4.60	5.00	2	2	2	40.6
F9UL68	Aminopeptidase C	4.55	5.87	2	2	2	50.2
F9UR64	NADH peroxidase	4.45	5.32	2	2	2	48.2
F9UUJ4	Transcription regulator, LacI family	4.41	9.54	2	2	2	35.0
Q9RE01	Uracil phosphoribosyltransferase	4.41	8.61	2	2	2	23.0
F9UUF0	Beta-galactosidase	4.32	5.96	2	2	2	35.2
F9ULF1	Transcription regulator, AraC family, GlcNAc-like induced	4.26	9.57	3	3	3	37.9
F9UL10	Cell surface protein, ErfK/YbiS/YcfS/YnhG family	4.25	4.96	2	2	2	51.1
F9UPM1	RNA-binding protein	4.22	8.47	2	2	2	33.7
F9UU32	3-hydroxy-3-methylglutaryl coenzyme A reductase	4.19	5.90	2	2	2	45.5
F9UQS7	UDP-N-acetylmuramyl tripeptide synthase	4.01	4.02	2	2	2	49.7
Q88XZ0	30S ribosomal protein S12	3.91	18.98	2	2	2	15.2
F9UTB5	Cystine ABC transporter, substrate binding protein	3.90	7.87	2	2	2	29.0
Q88Z86	Bifunctional protein GlmU	3.85	4.35	2	2	3	50.0
F9UU99	Glutamate decarboxylase	3.72	4.90	2	2	2	53.5
Q6LWI3	Replication protein	3.61	12.54	3	3	3	37.4
Q88XY7	30S ribosomal protein S10	3.22	21.57	2	2	2	11.7
F9UTV3	Multicopper oxidase	3.02	7.39	3	3	3	56.8
F9USG8	Short-chain dehydrogenase/oxidoreductase,classical SDR family, subgroup 1	2.98	6.08	2	2	2	31.5
F9UKV2	Purine biosynthesis operon repressor,phosphoribosyltransferase	2.74	8.27	2	2	2	30.5
Q88VD5	30S ribosomal protein S15	2.71	17.98	2	2	2	10.5
F9UP85	Maltose phosphorylase	2.49	2.94	2	2	2	85.6
F9UNZ9	Phosphatase, dihydroxyacetone kinase family	2.42	3.87	2	2	2	60.7
Q88XX7	30S ribosomal protein S17	2.30	20.22	2	2	2	10.3
Q88WJ1	50S ribosomal protein L19	2.27	14.41	2	2	3	13.6
Q88WU8	Translation initiation factor IF-3	2.12	12.72	2	2	3	19.6
Q88VE1	Trigger factor	1.74	4.09	2	2	2	49.4

Band # 7

Accession	Description	Score	Coverage	# Unique Peptides	# Peptides	# PSMs	MW [kDa]
Q88XZ3	DNA-directed RNA polymerase subunit beta	279.29	47.96	60	60	104	134.4
Q88XZ2	DNA-directed RNA polymerase subunit beta'	169.74	44.85	49	49	72	135.2
F9UPM3	Pyruvate kinase	130.26	52.22	31	31	48	62.8
Q88YM5	60 kDa chaperonin	107.46	53.05	29	29	43	57.4
Q88XY8	Elongation factor G	78.44	34.81	21	21	31	77.0
Q88WJ9	Chromosome partition protein Smc	74.69	20.59	20	20	24	131.8
F9UM10	Glyceraldehyde 3-phosphate dehydrogenase	63.92	42.35	13	13	21	36.4
Q88U41	ATP-dependent helicase/nuclease subunit A	63.25	15.85	17	17	22	140.9

Q88VM0	Chaperone protein DnaK	59.15	29.74	16	16	20	66.7
Q88VE0	Elongation factor Tu	58.05	35.70	15	15	31	43.4
F9URD9	Cell wall hydrolase/muramidase	57.48	24.46	13	13	21	82.1
Q88VJ2	D-lactate dehydrogenase	52.72	53.01	17	17	22	37.2
P77886	Carbamoyl-phosphate synthase pyrimidine-specific large chain	50.77	18.71	18	18	22	115.7
F9ULD9	ATP-dependent Clp protease, ATP-binding subunit ClpL	46.75	25.00	14	14	15	77.7
F9UQ80	Pyruvate carboxylase	45.75	16.54	15	15	18	127.1
F9UQ86	Translation elongation factor, GTPase,TypA/BipA family	45.40	26.96	15	15	19	68.4
Q88V10	Alanine--tRNA ligase	44.54	21.82	16	16	21	97.2
F9UPA5	Transpeptidase-transglycosylase (Penicillin binding protein 1A)	44.14	22.56	17	17	19	83.1
Q88YH3	Enolase 1	42.36	39.82	13	13	17	48.0
F9UMJ6	ATP-dependent Clp protease, ATP-binding subunit ClpC	38.81	17.92	11	12	14	92.6
Q88YH5	Phosphoglycerate kinase	36.17	35.00	11	11	15	42.8
F9UTT2	Fructose-bisphosphate aldolase	33.61	25.78	7	7	12	30.9
F9UPL0	30S ribosomal protein S1	32.66	36.13	15	15	18	47.1
F9US12	Cell surface protein, LPXTG-motif cell wall anchor	32.17	13.81	7	7	12	89.5
Q88VE1	Trigger factor	30.56	29.09	10	10	13	49.4
Q88V89	Isoleucine--tRNA ligase	29.40	12.55	12	12	14	106.3
Q88YL7	Protein translocase subunit SecA	28.58	13.72	9	9	10	89.5
Q88U67	Probable phosphoketolase 1	27.22	14.72	12	12	14	88.7
Q88YE7	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	24.16	15.37	8	8	10	65.4
F9UNZ9	Phosphatase, dihydroxyacetone kinase family	22.22	17.78	7	7	9	60.7
Q88VJ5	Elongation factor Ts	21.22	22.60	4	4	6	31.6
F9UNP5	DNA polymerase I	21.20	9.64	7	7	8	98.7
Q88YI7	UvrABC system protein A	20.62	10.20	8	8	9	104.9
F9UMD4	Aminopeptidase	20.15	14.22	11	11	11	93.9
Q88UX7	Valine--tRNA ligase	19.85	12.82	10	10	10	101.5
F9US18	Anaerobic ribonucleoside-triphosphate reductase	19.81	5.14	3	3	6	83.7
Q88VX7	Chaperone protein ClpB	19.27	9.34	6	7	8	96.5
F9UT05	Cell surface protein, LPXTG-motif cell wall anchor	18.41	6.36	5	5	8	105.1
F9UN58	ATP-dependent Clp protease, ATP-binding subunit ClpE	18.38	16.78	9	9	9	81.7
F9UNS5	6-phosphogluconate dehydrogenase, decarboxylating	17.93	14.02	5	5	7	52.9
F9UMH1	Asparagine synthase (Glutamine-hydrolysing)	17.75	14.85	9	9	9	73.1
F9UN60	Phosphoenolpyruvate-protein phosphotransferase	17.31	16.67	9	9	9	63.1
Q88Y74	GMP synthase [glutamine-hydrolyzing]	17.16	17.18	8	8	9	57.4
F9USY7	Maltodextrin-binding protein	16.74	12.95	4	4	6	45.6
Q88VS3	Glycine--tRNA ligase beta subunit	16.11	9.80	7	7	10	78.5
Q88W32	Probable manganese-dependent inorganic pyrophosphatase	15.99	12.62	3	3	5	33.6
F9UP42	Acetyl-CoA carboxylase, biotin carboxylase subunit	15.83	14.07	5	5	6	50.7
Q88VK1	Proline--tRNA ligase	15.68	15.29	8	8	9	63.5
F9ULZ0	Phosphoglucomutase	15.65	13.04	7	7	7	63.5
F9UMB1	DNA helicase	15.55	10.03	7	7	8	87.9
Q88WU9	Threonine--tRNA ligase	15.41	14.07	7	7	7	73.8

Q88V80	UDP-N-acetylmuramoylalanine--D-glutamate ligase	15.37	15.47	5	5	7	50.1
F9ULY4	UTP--glucose-1-phosphate uridylyltransferase	15.28	14.05	5	5	6	34.2
F9UR85	Aspartate-semialdehyde dehydrogenase	15.10	8.22	2	2	4	38.2
Q88Z76	CTP synthase	14.97	14.71	9	9	9	59.7
P56512	L-lactate dehydrogenase 1	14.96	26.56	7	7	8	34.2
Q88VJ4	30S ribosomal protein S2	14.63	27.72	7	7	7	30.2
Q88UU1	ATP synthase subunit alpha	14.22	15.08	7	7	7	54.5
F9UNT0	Exonuclease SbcC	13.85	4.52	4	4	5	117.9
Q88XB8	S-adenosylmethionine synthase	13.30	9.11	3	3	4	42.7
F9ULT0	Ribonucleoside-diphosphate reductase	12.70	6.80	4	4	5	82.1
Q88X53	Arginine--tRNA ligase	12.48	4.09	2	2	4	62.9
F9UT53	Cystathionine beta-lyase / cystathionine gamma-lyase	12.38	7.87	3	3	5	40.8
F9UL13	Transcription-repair-coupling factor	12.08	4.68	5	5	6	132.1
Q88YW9	50S ribosomal protein L1	11.76	18.78	4	4	4	24.8
F9UM05	Oligopeptide ABC transporter, substrate binding protein	11.58	9.45	5	5	5	61.0
Q88UZ4	Protein RecA	11.53	17.11	5	5	6	40.6
F9URQ9	Uncharacterized protein	11.50	8.93	5	5	6	76.2
F9UL89	Prophage P1 protein 2, mitogenic factor, cell surface lipoprotein	11.20	6.20	2	2	4	41.8
F9UP05	Signal recognition particle receptor FtsY	11.02	8.20	3	3	4	53.6
Q88VQ8	Aspartate--tRNA ligase	10.85	9.70	6	6	7	67.6
F9UUA0	Extracellular protein,gamma-D-glutamate-meso-diaminopimelate muropeptidase	10.76	11.08	3	3	3	36.8
F9UMU5	ATP-dependent DNA helicase	10.19	7.28	5	5	5	85.0
F9UTA3	GMP reductase	9.99	15.93	6	6	6	39.9
Q88YH4	Triosephosphate isomerase	9.94	20.24	4	4	4	27.0
Q88UI4	Glucose-6-phosphate isomerase	9.87	9.11	4	4	4	49.8
Q88XY3	50S ribosomal protein L2	9.87	24.01	6	6	7	30.2
Q9RE01	Uracil phosphoribosyltransferase	9.85	14.83	3	3	4	23.0
F9UP39	3-oxoacyl-[acyl-carrier-protein] synthase 2	9.69	6.59	2	2	3	42.4
Q88YW8	50S ribosomal protein L10	9.50	20.96	3	3	4	17.9
Q88UU3	ATP synthase subunit beta	9.44	7.71	3	3	3	50.8
F9UP45	Enoyl-[acyl-carrier-protein] reductase [NADH]	9.41	14.68	4	4	4	26.9
Q88Z97	Methionine--tRNA ligase	9.33	6.15	4	4	4	76.7
Q88XY0	30S ribosomal protein S3	8.44	25.81	5	5	6	24.2
F9UQC3	Cell division protein FtsZ	8.34	6.32	2	2	3	45.0
F9UN64	Serine-type D-Ala-D-Ala carboxypeptidase	8.23	12.62	4	4	4	45.2
F9UQ67	Ribonuclease J	7.92	6.10	3	3	3	63.9
F9UL68	Aminopeptidase C	7.79	8.80	4	4	4	50.2
Q6LWD9	Nickase	7.70	5.98	3	3	3	80.0
Q88VK7	Translation initiation factor IF-2	7.69	4.66	3	3	4	93.6
F9UP94	Aminotransferase	7.68	8.98	3	3	3	43.8
F9UNK2	FtsK/SpoIIIE family protein	7.63	5.32	5	5	5	103.7
F9UNI1	GTP cyclohydrolase-2	7.60	6.68	2	2	3	43.6
Q88WR2	Phenylalanine--tRNA ligase beta subunit	7.55	4.22	3	3	5	88.1
Q88Z86	Bifunctional protein GlmU	7.54	7.17	3	3	3	50.0
Q88UZ7	DNA mismatch repair protein MutS	7.54	5.02	4	4	4	99.9

F9UTG0	Glutathione reductase	7.43	6.76	3	3	4	48.2
Q88RX6	tRNA uridine 5-carboxymethylaminomethyl modification enzyme MnmG	7.10	3.93	3	3	4	70.7
Q88XY9	30S ribosomal protein S7	6.96	20.51	3	3	3	17.8
F9UL45	PTS system, mannose-specific EIIAB component	6.74	13.58	3	3	3	35.3
Q88XP7	Glutamyl-tRNA(Gln) amidotransferase subunit A	6.45	6.57	3	3	3	52.1
Q88XA9	Leucine--tRNA ligase	6.38	5.57	5	5	6	92.6
Q88WA8	Asparagine--tRNA ligase 2	6.35	7.87	4	4	4	49.8
F9UMX4	UDP-galactopyranose mutase	6.26	9.38	3	3	3	43.2
Q88YE8	Phosphoglucosamine mutase	6.12	9.09	3	3	3	48.3
F9UT98	Serine-type D-Ala-D-Ala carboxypeptidase	6.12	6.98	3	3	3	46.9
F9UU91	Cell surface protein, CscB family	6.07	2.73	2	2	2	118.7
Q88X05	Foldase protein PrsA 1	6.02	8.05	2	2	2	32.6
F9ULV5	ABC transporter, ATP-binding protein	5.95	4.24	2	2	2	74.1
F9URU9	Extracellular transglycosylase, with LysM peptidoglycan binding domain	5.85	10.45	3	3	3	35.0
Q88XZ7	Serine--tRNA ligase 2	5.81	5.65	2	2	2	48.1
F9US38	DNA gyrase subunit A	5.57	3.63	3	3	3	94.5
Q88VK2	DNA polymerase III PolC-type	5.57	2.57	3	3	4	161.9
F9UN23	Mannose-specific adhesin, LPXTG-motif cell wall anchor	5.47	4.65	3	3	3	107.9
F9UQM6	Cell shape determining protein MreB	5.36	8.68	3	3	3	35.1
F9UQP4	Universal stress protein	5.36	13.75	2	2	3	17.5
Q88XY6	50S ribosomal protein L3	5.11	17.22	3	3	3	22.7
F9UL33	N-acetylglucosamine-6-phosphate deacetylase	5.05	6.08	2	2	2	41.4
Q88XP6	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	4.97	9.07	4	4	4	53.2
F9UL55	Sigma54 activator, mannose PTS operon regulator	4.85	2.53	2	2	2	105.1
Q88Z31	ATP-dependent zinc metalloprotease FtsH	4.70	3.49	2	2	2	80.8
Q88WG0	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta 2	4.64	8.19	2	2	2	30.9
F9UM65	Pyruvate oxidase	4.55	3.07	2	2	2	63.6
Q88YW7	50S ribosomal protein L7/L12	4.51	31.97	3	3	3	12.6
Q88XX1	50S ribosomal protein L6	4.45	16.29	3	3	3	19.3
F9UQH6	Catabolite control protein A	4.44	9.23	3	3	3	36.3
F9UNV9	Glutamine synthetase	4.25	4.91	2	2	2	50.9
F9US93	Cell surface protein, LPXTG-motif cell wall anchor	4.00	5.35	2	2	3	64.8
F9UMJ0	Acetolactate synthase	3.96	3.57	2	2	2	61.2
Q88YY0	Glutamate--tRNA ligase	3.92	4.44	2	2	2	56.9
Q88TV5	Tyrosine--tRNA ligase	3.87	5.50	2	2	2	47.0
Q88V23	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase	3.74	10.17	2	2	3	24.5
F9UP38	3-oxoacyl-[acyl-carrier protein] reductase	3.67	7.02	2	2	2	25.7
F9UP07	Signal recognition particle protein	3.20	7.23	3	3	3	53.9
Q88XX4	50S ribosomal protein L5	3.13	15.56	3	3	3	20.2
F9UQZ2	Prophage P2a protein 4 AAA ATPase	2.94	4.64	2	2	2	66.5
Q88XW9	30S ribosomal protein S5	2.91	12.65	2	2	3	17.3
F9UT66	CDP-glycerol glycerophosphotransferase / glycosyltransferase	2.34	2.02	2	2	2	108.2

Q88T35	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2	2.26	10.00	2	2	2	26.1
Q88YD2	Polyphosphate kinase	2.09	2.92	2	2	2	82.2
Q88XW0	DNA-directed RNA polymerase subunit alpha	1.95	6.05	2	2	2	34.8
F9UR64	NADH peroxidase	0.00	5.32	2	2	3	48.2

Band # 8

Accession	Description	Score	Coverage	# Unique Peptides	# Peptides	# PSMs	MW [kDa]
Q88UX0	30S ribosomal protein S4	125.54	73.76	22	22	58	22.9
F9UM10	Glyceraldehyde 3-phosphate dehydrogenase	106.10	63.53	17	17	31	36.4
Q88XY5	50S ribosomal protein L4	101.59	57.00	14	14	33	22.6
Q88YH4	Triosephosphate isomerase	88.67	53.17	12	12	28	27.0
Q9RE01	Uracil phosphoribosyltransferase	83.81	47.37	10	10	26	23.0
Q88XY0	30S ribosomal protein S3	83.25	61.29	16	16	38	24.2
F9ULW6	Ribosome hibernation promoting factor	81.20	56.38	9	9	26	21.7
Q88XX4	50S ribosomal protein L5	75.48	73.89	13	13	27	20.2
F9UM84	Glutamine ABC transporter, ATP-binding protein	71.55	61.79	14	14	24	27.4
Q88XX1	50S ribosomal protein L6	70.01	50.00	9	9	27	19.3
Q88T35	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2	66.19	46.52	11	11	28	26.1
Q88YH9	ATP-dependent Clp protease proteolytic subunit	64.69	45.41	7	7	19	21.5
Q88VE0	Elongation factor Tu	64.47	47.59	15	15	24	43.4
F9UNS7	Two-component system response regulator	58.91	50.44	15	15	26	26.4
Q88VJ2	D-lactate dehydrogenase	56.95	44.58	16	16	21	37.2
F9UMA3	Phosphoglycerate mutase family protein	55.85	77.78	15	15	23	25.8
F9UL34	Transcription regulator, GntR family	54.17	61.37	12	12	18	26.3
Q88YW9	50S ribosomal protein L1	53.82	49.78	13	13	20	24.8
F9UTA0	Two component system response regulator	53.72	59.83	12	12	17	25.8
F9UP38	3-oxoacyl-[acyl-carrier protein] reductase	51.84	38.43	7	7	14	25.7
Q88VJ6	Uridylate kinase	51.82	52.08	11	11	17	25.9
F9UPM3	Pyruvate kinase	51.58	31.57	14	14	17	62.8
F9UPN0	S-adenosyl-L-methionine-dependent methyltransferase	49.75	71.49	13	13	16	25.6
Q88XW5	Adenylate kinase	49.50	64.38	13	13	21	24.4
F9UNK1	Phenylalanine--tRNA ligase beta subunit	48.26	36.15	5	5	14	23.0
Q88YH3	Enolase 1	46.95	31.67	10	10	14	48.0
F9UMV3	Transcription regulator, TetR family	45.74	37.70	7	7	14	21.6
Q88VJ7	Ribosome-recycling factor	45.69	52.94	8	8	14	20.6
F9UNA0	Deoxynucleoside kinase	43.31	41.86	7	7	13	24.9
F9UP44	Acetyl-CoA carboxylase, carboxyl transferase subunit alpha	43.10	53.13	9	9	13	27.6
F9UTT2	Fructose-bisphosphate aldolase	42.79	39.72	9	9	14	30.9
F9UMY6	dTDP-4-dehydrorhamnose 3,5-epimerase	42.58	50.78	10	10	15	21.8
Q88ZS6	Glucosamine-6-phosphate deaminase	42.22	56.12	11	11	15	25.9
Q88VJ4	30S ribosomal protein S2	41.56	41.20	11	11	16	30.2
Q88WN1	Elongation factor P	41.11	38.38	8	8	17	20.4
F9UQF7	Peptidyl-prolyl cis-trans isomerase	40.93	44.85	7	7	16	21.1

F9UMC8	Alkaline shock protein	40.89	62.14	8	8	14	15.8
Q88YM8	Redox-sensing transcriptional repressor Rex	40.83	30.67	11	11	16	25.2
Q88YH5	Phosphoglycerate kinase	40.47	37.50	13	13	15	42.8
F9UP45	Enoyl-[acyl-carrier-protein] reductase [NADH]	40.45	52.78	11	11	17	26.9
F9UM05	Oligopeptide ABC transporter, substrate binding protein	40.44	27.45	14	14	16	61.0
F9UN72	Acetyltransferase, GNAT family	39.62	47.09	5	5	13	19.2
P77888	Orotidine 5'-phosphate decarboxylase	38.71	38.40	7	7	14	24.9
F9UPJ6	Extracellular protein, DUF2140 family	38.29	34.58	7	7	13	23.7
F9URH4	Short-chain dehydrogenase, atypical SDR family,subgroup 5	38.13	56.34	9	9	12	22.9
F9UQB6	ADP-ribose pyrophosphatase	37.86	51.63	7	7	11	20.5
F9UPD3	Phosphoglycerate mutase family protein	37.63	63.32	10	10	13	22.6
Q88UU0	ATP synthase subunit delta	37.61	48.07	8	8	12	20.0
P77889	Orotate phosphoribosyltransferase	36.56	48.58	8	8	13	22.7
F9UMI5	Transcriptional attenuator, cell envelope-related, LytR family	36.31	35.07	10	10	11	37.7
Q88XY6	50S ribosomal protein L3	36.30	41.15	8	8	12	22.7
P71479	Bifunctional protein PyrR 1	35.38	63.89	9	10	13	19.8
Q88XY3	50S ribosomal protein L2	34.63	59.86	13	13	17	30.2
F9UNZ2	Serine/threonine specific protein phosphatase	34.48	34.27	8	8	12	27.2
Q88WZ9	tRNA (guanine-N(7)-)-methyltransferase	33.31	37.85	7	7	12	24.6
F9UNT5	rRNA methylase, SpoU family	32.78	43.14	6	6	10	27.6
F9UM81	Phosphohydrolase	32.30	34.13	7	7	11	23.2
Q88W32	Probable manganese-dependent inorganic pyrophosphatase	32.11	30.74	10	10	12	33.6
Q88YW8	50S ribosomal protein L10	31.83	52.10	6	6	10	17.9
F9UQM0	Glutamine/histidine ABC transporter,ATP-binding protein	31.83	38.97	6	6	9	23.4
F9UMA4	Phosphoglycerate mutase family protein	31.33	48.62	7	7	9	24.4
Q88VZ4	Cytidylate kinase	30.33	33.77	6	6	9	24.6
Q88UT1	Thymidine kinase	29.70	48.44	12	12	15	22.1
Q88ST4	GTP cyclohydrolase 1	29.65	57.14	8	8	11	21.4
Q88XY9	30S ribosomal protein S7	29.53	60.90	8	8	12	17.8
F9URP2	Transcription regulator, TetR family	29.39	47.98	9	9	11	25.2
F9UUC8	Cell surface protein, CscD family,LPXTG-motif cell wall anchor	29.07	40.00	7	7	9	22.1
Q88YY5	Ribose-5-phosphate isomerase A	28.30	28.19	6	6	9	24.6
Q6LWD5	Putative resolvase (Fragment)	27.78	40.00	7	7	10	22.3
F9UL82	Transcription termination/antitermination protein NusG	27.28	32.97	5	5	10	20.6
F9UTA8	Chomosome partitioning protein,membrane-associated ATPase	27.26	32.55	6	6	8	27.8
F9URD0	Lipase/esterase	27.23	26.05	5	5	9	28.3
F9UNZ5	Ribulose-phosphate 3-epimerase	27.15	31.80	6	6	9	23.4
F9URA8	Transaldolase O	26.61	30.80	7	7	9	25.8
F9URR4	3-dehydroquinate dehydratase	26.52	41.77	7	7	8	26.6
Q88X05	Foldase protein PrsA 1	26.45	29.19	8	8	10	32.6
Q88YP6	Thymidylate kinase	26.32	45.70	10	10	12	24.3
F9UMI1	Metal-dependent regulator	26.21	40.00	8	8	9	23.9
F9URS2	Extracellular protein	26.17	33.48	5	5	7	23.2
F9UQA4	Uncharacterized protein	25.97	29.46	6	6	10	25.4
F9URW9	Phosphoglycerate mutase family protein	25.58	31.60	5	5	8	22.9

F9UQM3	Septum site-determining protein MinC	25.46	33.33	7	7	9	24.7
F9UTV2	Ribose 5-phosphate epimerase	25.34	34.23	5	5	8	23.9
Q88XY8	Elongation factor G	25.33	10.32	6	6	8	77.0
F9US84	Beta-phosphoglucomutase	24.92	33.18	6	6	10	23.7
F9URQ8	Oxidoreductase	24.69	39.47	5	5	10	20.4
F9UU90	Extracellular protein, DUF1002 family	24.18	28.26	5	5	6	34.2
Q88WK0	Ribonuclease 3	23.90	27.27	7	7	9	26.6
Q88XW9	30S ribosomal protein S5	23.71	46.99	7	7	8	17.3
F9ULY5	Diguanylate cyclase/phosphodiesterase, EAL domain	23.56	34.51	9	9	11	26.2
F9US66	Serine protease HtrA	22.95	22.62	6	6	7	43.1
F9ULZ5	Metal-dependent phosphohydrolase, HD family	22.74	41.40	9	9	9	24.5
Q88VB2	Peptide deformylase	22.61	24.73	4	4	7	20.8
F9US95	Lipase/esterase, subfamily of SGNH-hydrolases	22.30	33.05	6	6	8	25.5
F9UL32	Pyrroline-5-carboxylate reductase	22.29	25.86	5	5	8	27.0
F9UNV2	Bifunctional protein: transcription regulator sugar kinase, ROK family	22.11	24.69	6	6	7	33.7
F9UR12	Prophage P2b protein 2, phage transcription regulator, Cro/CI family OS=Lactobacillus plantarum (strain ATCC BAA-793 / NCIMB 8826 / WCFS1) OX=220668 GN=lp_2479 PE=4 SV=1 - [F9UR12_LACPL]	21.99	32.98	7	7	8	21.7
F9UMJ3	Deoxyadenosine kinase / deoxyguanosine kinase	21.71	27.96	5	5	7	24.3
F9URN3	Phosphohydrolase, MutT/nudix family	21.63	40.43	5	5	8	20.7
Q6LWI0	Replication protein	21.61	33.79	7	7	8	25.3
Q88WL7	Guanylate kinase	21.32	34.47	7	7	9	23.4
F9UQ57	Glutamine ABC transporter, ATP-binding protein	21.22	28.46	5	5	7	26.9
Q88V20	dITP/XTP pyrophosphatase	21.14	53.47	10	10	10	21.8
F9UP96	DNA replication protein DnaD	21.14	22.82	5	5	7	27.4
F9ULV1	Glycoprotein endopeptidase, M22 family	21.12	39.42	7	7	9	26.2
F9ULT2	16S rRNA methyltransferase	21.02	37.13	7	7	8	22.1
Q88WA8	Asparagine--tRNA ligase 2	20.48	21.30	7	7	7	49.8
Q88YP8	Recombination protein RecR	20.05	44.22	7	7	7	21.9
F9UL91	Prophage P1 protein 5, superinfection exclusion (Cell surface N-anchored)	19.88	13.22	5	5	8	37.9
F9UL19	Hypoxanthine phosphoribosyltransferase	19.58	35.56	7	7	10	20.3
Q88WG0	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta 2	19.48	26.69	6	6	7	30.9
F9URR0	Flavodoxin	19.48	47.30	6	6	7	16.0
F9UQP4	Universal stress protein	19.48	48.13	5	5	6	17.5
Q88XY4	50S ribosomal protein L23	19.10	46.39	4	4	5	11.1
F9US56	Beta-phosphoglucomutase	18.90	27.80	6	6	8	23.6
Q88XU8	50S ribosomal protein L13	18.36	40.82	5	5	6	16.2
F9UQI5	Phosphoesterase	18.31	31.40	5	5	9	19.0
F9UMG5	Phosphoglycerate mutase family protein	18.30	25.23	5	5	7	24.5
F9URW1	Endonuclease III	17.97	17.59	3	3	6	23.9
Q890D7	UPF0246 protein lp_0089	17.94	28.63	5	5	6	28.6
F9UL77	tRNA/rRNA methyltransferase, TrmH family	17.72	29.80	6	6	6	27.5
Q88YW7	50S ribosomal protein L7/L12	17.68	53.28	6	6	6	12.6
F9USU0	Glutamine amidotransferase, class I	17.67	24.80	5	5	6	26.2
Q88ZA0	NAD-dependent protein deacetylase	17.56	28.21	5	5	6	26.4

F9UL09	Uncharacterized protein	17.51	33.02	7	7	8	23.9
F9UMU7	Lipoprotein, pheromone	17.48	13.55	4	4	6	42.6
Q88UI4	Glucose-6-phosphate isomerase	17.15	15.11	6	6	7	49.8
Q88XQ4	Xanthine phosphoribosyltransferase	16.98	23.59	4	4	7	21.4
Q88VJ5	Elongation factor Ts	16.94	24.32	6	6	6	31.6
F9USY7	Maltodextrin-binding protein	16.84	16.79	5	5	6	45.6
F9UTM7	Short-chain dehydrogenase/oxidoreductase,atypical SDR family, subgroup 6	16.71	26.89	6	6	6	23.5
F9UQE4	Copper homeostasis protein CutC	16.53	33.96	4	4	4	22.4
F9UQA5	Phosphoglycerate mutase family protein	16.41	32.58	5	5	5	24.9
Q88VM0	Chaperone protein DnaK	16.38	10.61	5	5	5	66.7
F9URF5	Two-component system response regulator	16.29	26.51	6	6	8	28.5
F9ULS5	DNA entry nuclease	16.19	14.77	4	4	5	36.2
F9UKV8	Hydrolase, HAD superfamily, Cof family	16.12	24.54	5	5	7	29.4
Q88YY8	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 1	15.99	36.89	5	5	6	25.6
F9US55	Hydrolase, HAD superfamily, Cof family	15.79	23.53	5	5	7	28.7
F9UL46	PTS system, mannose-specific EIIC component	15.65	8.58	2	2	3	27.2
F9UM18	Carboxylesterase	15.51	20.48	3	3	5	27.9
F9URY3	NADP oxidoreductase, coenzyme F420-dependent	15.34	42.64	7	7	10	21.0
F9UTU3	Transport permease protein	15.34	16.24	5	5	6	31.7
F9UT12	Phosphoglycerate mutase family protein	15.32	38.65	7	7	8	23.0
Q88WU6	50S ribosomal protein L20	15.22	40.68	5	5	6	13.6
Q88Y74	GMP synthase [glutamine-hydrolyzing]	15.10	11.78	5	5	5	57.4
F9UQD7	NADH-flavin reductase	15.02	14.42	3	3	5	23.0
F9UTC8	ABC transporter, ATP-binding protein	14.99	30.56	6	6	8	24.0
F9UNJ6	ABC transporter, ATP-binding protein	14.92	22.45	3	3	5	26.7
F9UPK7	DNA-binding protein	14.92	67.03	4	4	4	9.6
F9UMY8	dTDP-4-dehydrorhamnose reductase	14.46	24.29	5	5	6	31.3
F9UQB4	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase	14.38	21.30	3	3	4	24.5
F9UTA3	GMP reductase	14.29	18.28	5	5	6	39.9
F9US28	Uncharacterized protein	14.28	10.58	4	4	4	53.3
F9UPW1	Uncharacterized protein	14.23	35.14	6	6	6	24.9
Q890J8	50S ribosomal protein L9	14.00	36.67	5	5	5	16.5
F9UNB8	Uncharacterized protein	13.97	28.71	5	5	5	23.2
F9UT53	Cystathionine beta-lyase / cystathionine gamma-lyase	13.91	16.54	5	5	5	40.8
F9URN0	Hydrolase, HAD superfamily, Cof family	13.78	22.64	5	5	5	29.2
Q88Y41	FMN-dependent NADH-azoreductase 2	13.71	32.06	5	5	7	23.6
F9US11	Transcription regulator, GntR family	13.68	25.94	5	5	5	27.2
F9UT98	Serine-type D-Ala-D-Ala carboxypeptidase	13.64	9.68	3	3	4	46.9
F9USN8	DNA-binding ferritin-like protein, DPS family	13.61	25.81	4	4	5	18.0
Q88VE3	Probable GTP-binding protein EngB	13.26	25.89	5	5	5	22.4
F9UNZ6	Thiamin pyrophosphokinase	13.24	24.66	3	3	4	24.1
Q88YM5	60 kDa chaperonin	13.21	8.32	3	3	5	57.4
Q88WR0	Uridine kinase	12.70	24.40	4	4	4	24.0
F9UTF3	DegV family protein	12.59	21.51	4	4	4	29.6
F9UNC1	Two-component system response regulator	12.54	22.35	5	5	5	29.2
Q88WT5	Probable nicotinate-nucleotide adenylyltransferase	12.33	26.54	5	5	5	24.0

F9UL67	Extracellular zinc metalloproteinase, M10 family	12.33	30.00	5	5	6	26.5
F9URF4	Metal-dependent phosphohydrolase, HD family	12.18	26.15	5	5	6	24.3
F9ULD5	Two-component system response regulator accesory gene regulator protein A	12.10	22.27	5	5	7	28.6
F9UR85	Aspartate-semialdehyde dehydrogenase	11.96	14.73	4	4	4	38.2
Q88U67	Probable phosphoketolase 1	11.96	4.31	3	3	4	88.7
F9UUB9	Transcription regulator, Crp family	11.62	22.62	4	4	4	24.5
F9USK4	Transcription regulator, TetR family	11.38	26.42	4	4	4	21.4
F9UP25	Isochorismatase	11.10	15.64	2	2	3	19.8
F9UPF5	Lipoprotein	11.04	17.69	4	4	4	31.0
Q88XW7	50S ribosomal protein L15	11.02	33.57	4	4	4	15.3
F9URN1	Uncharacterized protein	10.98	21.33	4	4	4	23.9
F9USG8	Short-chain dehydrogenase/oxidoreductase,classical SDR family, subgroup 1	10.83	11.15	3	3	4	31.5
F9UMX8	Glycosyltransferase	10.81	17.05	3	3	4	30.4
F9UM37	Diadenylate cyclase	10.55	15.36	4	4	4	31.5
F9USK1	Two-component system response regulator	10.51	20.16	4	4	4	28.6
F9UNS5	6-phosphogluconate dehydrogenase, decarboxylating	10.36	10.67	4	4	4	52.9
Q88XV9	50S ribosomal protein L17	10.29	29.13	3	3	3	14.2
F9UN60	Phosphoenolpyruvate-protein phosphotransferase	10.15	7.47	3	3	3	63.1
F9ULZ0	Phosphoglucomutase	10.13	9.22	4	4	4	63.5
P56512	L-lactate dehydrogenase 1	10.13	17.50	6	6	6	34.2
F9URB8	ABC transporter, ATP-binding protein	10.10	13.14	2	2	3	26.4
F9UQG3	Metallophosphatase	10.06	20.30	3	4	4	23.4
Q88YZ2	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta 1	10.05	12.36	3	3	3	29.2
Q88XX2	30S ribosomal protein S8	9.93	25.19	3	3	4	14.6
F9UM77	Guanylate kinase	9.90	26.42	5	5	5	21.7
F9UTR7	NADH dehydrogenase	9.89	14.75	4	4	5	43.8
F9UT87	Cyclopropane-fatty-acyl-phospholipid synthase	9.80	11.84	4	4	6	45.3
F9UL47	PTS system, mannose-specific EIID component	9.80	13.11	3	3	3	33.6
F9UM41	Diguanylate cyclase/phosphodiesterase, EAL domain	9.72	19.73	3	3	3	25.4
F9UQS8	Glutamine amidotransferase, CobQ-like domain	9.71	21.25	3	3	3	26.6
F9UQA9	Potassium uptake protein	9.67	16.36	4	4	4	24.3
F9UQM5	Cell shape-determining protein MreC	9.65	18.15	4	4	4	30.1
Q88UW9	UPF0637 protein lp_2332	9.59	16.83	3	3	3	23.5
Q88VI6	LexA repressor	9.49	21.90	3	3	4	23.3
Q88WQ9	Transcription elongation factor GreA 2	9.48	30.63	5	5	6	17.9
F9UPH4	Transcription regulator, LysR family	9.37	11.91	3	3	3	36.8
Q88XW0	DNA-directed RNA polymerase subunit alpha	9.33	6.69	2	2	3	34.8
F9UQH9	Extracellular protein, DUF336 family	9.27	27.06	4	4	4	18.0
F9UMC9	Alkaline shock protein	9.21	26.71	3	3	3	16.1
F9UQM2	Site-determining protein	9.16	10.07	2	2	2	29.1
F9UQF4	Uncharacterized protein	9.14	29.52	4	4	4	19.2
F9UNI1	GTP cyclohydrolase-2	9.11	12.38	4	4	4	43.6
F9UM31	Exodeoxyribonuclease III	9.11	20.08	4	4	4	29.6

F9UPV2	Ribosomal RNA small subunit methyltransferase E	9.06	18.00	3	3	4	26.9
F9UM23	Glutamine ABC transporter, ATP-binding protein	9.03	17.62	4	4	4	26.8
Q88YK2	Prolipoprotein diacylglyceryl transferase	9.00	11.35	3	3	3	32.3
F9UM38	Cell surface protein, YbbR-like family	8.97	12.31	3	3	3	34.6
F9UT55	Methionine aminopeptidase	8.93	17.49	3	3	3	29.1
F9ULD9	ATP-dependent Clp protease, ATP-binding subunit ClpL	8.88	5.26	3	3	4	77.7
F9US18	Anaerobic ribonucleoside-triphosphate reductase	8.85	5.54	4	4	4	83.7
Q88W05	Thymidylate synthase	8.81	6.96	2	2	3	36.0
F9UPC1	ABC transporter, ATP-binding protein	8.71	12.44	2	2	3	25.6
F9USU9	NADPH-dependent FMN reductase family protein	8.69	12.00	2	2	4	22.2
F9ULZ1	Diguanylate cyclase/phosphodiesterase, EAL domain	8.68	25.42	6	6	6	27.1
F9USG6	Extracellular zinc metalloproteinase	8.66	11.05	3	3	3	38.2
F9URT4	Dihydrofolate reductase family protein	8.65	19.55	4	4	4	20.1
F9UQ34	Transcription regulator	8.59	12.89	3	3	4	21.9
P59389	Bifunctional protein PyrR 2	8.57	17.82	2	3	3	19.3
Q88WN5	50S ribosomal protein L21	8.51	20.59	2	2	3	11.0
F9UQZ0	Prophage P2a protein 6 endonuclease	8.48	9.07	3	3	3	40.6
Q88YI4	Nucleotide-binding protein lp_0779	8.45	12.59	3	3	3	33.3
F9USL6	Transcription regulator, TetR family	8.41	10.05	2	2	3	22.6
F9UL45	PTS system, mannose-specific EIIAB component	8.40	8.95	3	3	3	35.3
Q88WG9	3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ	8.39	21.09	3	3	3	16.0
Q88XW2	30S ribosomal protein S13	8.34	32.23	4	4	4	13.6
F9UPN8	Lipoprotein	8.34	9.97	4	4	4	41.6
F9UQS7	UDP-N-acetylmuramyl tripeptide synthase	8.33	9.60	4	4	4	49.7
F9URN5	3-ketoacyl-(Acyl-carrier-protein) reductase	8.24	20.97	4	4	4	25.8
Q88WU8	Translation initiation factor IF-3	8.17	20.23	3	3	3	19.6
Q88WJ1	50S ribosomal protein L19	8.14	31.36	4	4	5	13.6
F9UTN1	Transcription regulator, TetR family	8.13	16.16	4	4	4	25.4
F9ULL2	Phenolic acid decarboxylase	8.05	16.29	3	3	5	21.0
Q88RX1	Membrane protein insertase YidC 2	7.93	9.03	2	2	2	31.7
F9USJ4	(Pyro)phosphohydrolase, HAD superfamily	7.92	15.67	4	4	4	24.1
F9UR42	Phosphohydrolase, MutT/nudix family	7.91	16.19	3	3	3	28.0
Q890K2	30S ribosomal protein S6	7.88	37.37	3	3	3	11.4
F9UNW6	Transcription regulator, TetR family	7.86	18.18	3	3	3	24.0
F9URL0	Carbonate dehydratase	7.84	17.54	5	5	5	23.4
F9UTS6	Acetyltransferase, GNAT family	7.81	14.86	2	2	2	19.7
F9USJ9	Cell surface protein, ErfK family	7.72	17.22	3	3	3	23.5
Q88V23	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase	7.68	19.92	4	4	5	24.5
F9UQQ1	L-2-hydroxyisocaproate dehydrogenase	7.57	12.13	3	3	3	32.5
Q88WU9	Threonine--tRNA ligase	7.54	6.12	3	3	4	73.8
F9UP75	1,4-dihydroxy-2-naphthoate octaprenyltransferase, UbiA prenyltransferase family	7.44	5.90	2	2	3	34.4
F9USU4	Uncharacterized protein	7.37	11.06	3	3	3	26.9
Q88XW1	30S ribosomal protein S11	7.34	21.71	2	2	2	13.8
F9UQ58	Glutamine ABC transporter, substrate binding and permease protein	7.33	6.31	3	3	3	53.8

Q88YE8	Phosphoglucosamine mutase	7.31	6.43	2	2	2	48.3
F9UP42	Acetyl-CoA carboxylase, biotin carboxylase subunit	7.27	11.47	4	4	4	50.7
F9UMU5	ATP-dependent DNA helicase	7.26	3.97	3	3	4	85.0
F9UMJ7	Transcription regulator, TetR family	7.19	16.00	2	2	2	22.4
F9UQV2	Prophage P2a protein 45	7.18	16.49	3	3	3	20.1
Q88YI6	S-ribosylhomocysteine lyase	6.99	15.82	2	2	2	17.4
F9UT06	Transcription regulator, Xre family	6.97	12.14	3	3	3	32.0
Q88VM6	D-alanine--D-alanyl carrier protein ligase	6.94	8.86	3	3	4	56.0
F9UN64	Serine-type D-Ala-D-Ala carboxypeptidase	6.78	6.31	2	2	2	45.2
F9UNZ3	Serine/threonine protein kinase	6.74	3.71	2	2	2	74.5
F9UNS3	Lipoprotein	6.74	32.47	5	5	5	21.1
F9UP39	3-oxoacyl-[acyl-carrier-protein] synthase 2	6.73	8.54	3	3	3	42.4
F9USN1	Transcription regulator, TetR family	6.58	19.09	4	4	4	25.3
Q88WV3	Dephospho-CoA kinase	6.58	16.75	3	3	3	21.3
F9URQ0	Transcription regulator, GntR family	6.54	11.91	2	2	2	27.0
Q88YK1	Glycerol-3-phosphate dehydrogenase [NAD(P)+]	6.49	14.50	3	3	3	36.6
Q88SK7	Undecaprenyl-diphosphatase	6.41	10.91	3	3	4	30.5
F9UTM5	Small heat shock protein	6.31	21.09	2	2	2	16.7
F9UPP8	Phosphohydrolase, MutT/nudix family	6.25	9.95	2	2	3	22.3
F9ULV2	Ribosomal-protein-alanine N-acetyltransferase	6.20	12.50	2	2	3	22.6
F9URM9	Threonine synthase	6.15	6.04	2	2	2	54.5
Q88T16	Foldase protein PrsA 2	6.14	6.47	2	2	3	34.3
Q88XW3	50S ribosomal protein L36	6.06	58.97	2	2	3	4.6
Q88XZ0	30S ribosomal protein S12	6.01	8.76	2	2	4	15.2
Q88WV2	Transcriptional repressor NrdR	5.93	16.37	2	2	2	19.9
F9US17	Anaerobic ribonucleoside-triphosphate reductase-activating protein	5.84	16.06	4	4	4	22.0
Q88VS2	Glycine--tRNA ligase alpha subunit	5.78	8.70	2	2	2	34.5
F9UQB9	Cell division initiation protein DivIVA	5.71	16.38	3	3	3	26.2
F9UU94	Transcription regulator, AraC family	5.60	11.02	2	2	2	29.3
Q88YG1	Uracil-DNA glycosylase	5.54	8.26	2	2	2	26.1
F9UMG4	Acetyltransferase, GNAT family	5.48	11.11	2	2	3	20.5
F9UMT3	Cytochrome D ubiquinol oxidase, subunit II	5.47	6.34	2	2	2	37.3
F9UNH2	NADPH-dependent FMN reductase family protein	5.45	11.27	2	2	2	22.9
F9US91	Glutamine amidotransferase class-I	5.45	18.02	3	3	3	25.0
Q88XY7	30S ribosomal protein S10	5.31	31.37	3	3	3	11.7
F9USH9	Transcription regulator, AraC family	5.28	7.91	2	2	2	29.6
F9USR4	ThiJ/PfpI family protein, Type 1 glutamine amidotransferase (GATase1)-like domain	5.27	13.39	2	2	2	24.8
F9UTB5	Cystine ABC transporter, substrate binding protein	5.17	11.61	3	3	3	29.0
F9UNU9	5-formyltetrahydrofolate cyclo-ligase	5.15	13.30	2	2	2	21.4
Q88VQ7	Histidine--tRNA ligase	5.10	5.40	2	2	2	47.9
F9UQC9	Transpeptidase, penicillin binding protein 2B	5.10	3.37	2	2	2	77.2
F9UPP7	Transcription regulator, DeoR family	5.06	11.60	3	3	3	28.0
Q88W97	Holliday junction resolvase RecU	5.01	11.59	3	3	3	23.7
F9UQL5	Transcription regulator, Xre family	5.01	11.72	2	2	2	31.3
P77883	Aspartate carbamoyltransferase	4.98	7.72	2	2	2	34.7

F9UT61	PTS system, trehalose-specific IIBC component	4.96	3.46	2	2	2	69.4
F9UL78	Nuclease, NYN_YacP family	4.94	9.94	2	2	2	21.0
F9UTN9	Two-component system response regulator	4.92	11.54	2	2	2	26.8
F9UQM6	Cell shape determining protein MreB	4.85	8.68	3	3	4	35.1
Q88VJ8	Ditrans,polycis-undecaprenyl-diphosphate synthase ((2E,6E)-farnesyl-diphosphate specific)	4.81	8.11	2	2	2	28.8
Q890C0	Thiamine-phosphate synthase	4.81	11.52	2	2	2	22.8
F9USU5	Alpha-beta hydrolase superfamily	4.80	13.08	3	3	3	29.0
F9UKZ1	Sortase A	4.76	10.68	2	2	2	26.0
F9UPK2	Uncharacterized protein	4.75	12.67	2	2	3	17.1
F9UQF3	Metal-dependent hydrolase, beta-lactamase superfamily III	4.68	6.48	2	2	3	26.8
Q890C2	Hydroxyethylthiazole kinase	4.64	9.13	2	2	2	27.5
Q88XX7	30S ribosomal protein S17	4.62	20.22	2	2	2	10.3
F9UPC2	Hypothetical membrane protein	4.62	5.57	2	2	2	41.3
F9UQQ0	Uncharacterized protein	4.62	7.10	2	2	2	36.8
F9UTJ4	Beta-lactamase family protein	4.57	3.65	2	2	3	37.3
F9UL33	N-acetylglucosamine-6-phosphate deacetylase	4.56	6.08	2	2	2	41.4
F9UPB8	Glycosyl hydrolase, family 25	4.55	10.96	3	3	3	50.0
Q88UU1	ATP synthase subunit alpha	4.47	4.17	2	2	2	54.5
F9UPD5	Chloramphenicol O-acetyltransferase	4.20	9.01	2	2	2	25.4
F9ULT0	Ribonucleoside-diphosphate reductase	4.19	3.74	2	2	2	82.1
F9UQ97	Ribonuclease J	3.75	5.56	2	2	2	61.8
F9UMX4	UDP-galactopyranose mutase	3.71	7.77	3	3	3	43.2
F9UL62	Acetyl-CoA carboxylase, carboxyl transferase subunit alpha	3.54	8.95	2	2	2	27.3
F9UT59	Trehalose operon transcriptional repressor,GntR family	3.48	9.21	2	2	2	26.9
Q88XX9	50S ribosomal protein L16	3.17	17.36	2	2	2	16.1
F9URK7	NADPH-dependent FMN reductase family protein	3.09	8.42	2	2	2	22.8
F9UL42	Homoserine dehydrogenase	3.05	4.44	2	2	2	46.6
F9UMH1	Asparagine synthase (Glutamine-hydrolysing)	2.83	4.74	2	2	2	73.1
Q88YM6	10 kDa chaperonin	2.74	17.02	2	2	2	10.3
F9UMY7	dTDP-glucose 4,6-dehydratase	2.73	4.68	2	2	2	38.6
F9UQK0	Preprotein translocase, YajC subunit	2.65	18.11	2	2	2	13.8
F9UPL0	30S ribosomal protein S1	2.64	4.43	2	2	2	47.1
Q88XX0	50S ribosomal protein L18	2.64	13.56	2	2	2	13.0
F9ULY7	Thioredoxin reductase	2.61	6.41	2	2	2	33.4
Q88VD5	30S ribosomal protein S15	2.60	17.98	2	2	2	10.5
F9UT13	Oxidoreductase, aldo/keto reductase family	2.59	7.60	2	2	2	29.3
Q88XZ3	DNA-directed RNA polymerase subunit beta	2.40	1.67	2	2	2	134.4
Q88YF4	UDP-N-acetylenolpyruvoylglucosamine reductase	2.40	7.95	2	2	2	32.3
Q88VQ8	Aspartate--tRNA ligase	2.39	5.02	4	4	4	67.6
F9UPA5	Transpeptidase-transglycosylase (Penicillin binding protein 1A)	2.34	2.74	2	2	2	83.1
F9UQH7	Xaa-Pro dipeptidase	2.23	5.15	2	2	2	41.1
Q88Z84	Ribose-phosphate pyrophosphokinase 1	2.23	6.44	2	2	2	35.9
F9ULS9	Ribonucleoside-diphosphate reductase, beta chain	2.17	9.23	3	3	3	39.1
F9UT44	Transcription regulator, GntR family	2.15	11.26	2	2	2	26.2

Q88X16	6,7-dimethyl-8-ribityllumazine synthase	2.12	11.95	2	2	2	16.7
F9UN65	Aminotransferase	2.10	9.62	3	3	3	43.8
F9UTE1	Endonuclease III	1.97	11.71	3	3	3	25.5
F9UTK1	Uncharacterized protein	1.88	19.01	3	3	3	16.7
F9UTW0	Purine nucleosidase	0.00	6.86	2	2	2	33.0
F9UTY4	Galactoside O-acetyltransferase	0.00	15.12	2	2	2	23.1
F9URU0	Phosphohydrolase, HAD superfamily	0.00	13.64	2	2	2	25.4
F9URL3	ABC transporter, ATP-binding protein	0.00	5.16	1	2	2	27.7

Band # 9

Accession	Description	Score	Coverage	# Unique Peptides	# Peptides	# PSMs	MW [kDa]
Q88YW7	50S ribosomal protein L7/L12	51.64	72.13	11	11	17	12.6
Q88XY4	50S ribosomal protein L23	50.82	78.35	12	12	18	11.1
Q88XV9	50S ribosomal protein L17	50.17	57.48	7	7	16	14.2
F9USN8	DNA-binding ferritin-like protein, DPS family	46.07	39.35	6	6	15	18.0
Q88WN5	50S ribosomal protein L21	45.67	65.69	7	7	14	11.0
Q88WG9	3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ	45.13	51.02	9	9	16	16.0
Q88RX0	Ribonuclease P protein component	45.12	63.16	8	8	15	13.2
Q88XW1	30S ribosomal protein S11	42.67	32.56	4	4	12	13.8
Q88WU6	50S ribosomal protein L20	39.70	38.98	6	6	14	13.6
Q88YW9	50S ribosomal protein L1	37.22	32.31	9	9	12	24.8
F9US66	Serine protease HtrA	37.18	28.10	9	9	11	43.1
Q88XY1	50S ribosomal protein L22	36.82	43.48	7	7	13	12.5
F9UU90	Extracellular protein, DUF1002 family	36.31	24.53	6	6	9	34.2
Q88XY9	30S ribosomal protein S7	35.97	55.13	9	9	15	17.8
Q88XX2	30S ribosomal protein S8	35.81	55.73	7	7	12	14.6
Q88WJ1	50S ribosomal protein L19	34.85	51.69	10	10	20	13.6
F9UPV6	Uncharacterized protein	33.10	59.38	6	6	12	11.0
F9UM10	Glyceraldehyde 3-phosphate dehydrogenase	32.09	27.94	10	10	11	36.4
Q88W95	Cell cycle protein GpsB	30.58	48.67	6	6	11	12.9
F9UP41	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase	29.24	45.99	6	6	11	15.1
Q88XW2	30S ribosomal protein S13	28.77	52.07	8	8	13	13.6
Q88YH3	Enolase 1	28.69	22.17	7	7	10	48.0
F9UQ60	Uncharacterized protein	28.67	51.11	7	7	10	14.5
Q88X05	Foldase protein PrsA 1	28.32	27.52	7	7	9	32.6
F9UPJ4	Carboxy-terminal proteinase, S41 family,peptidoglycan-bound	28.12	21.75	10	10	12	53.2
Q88VE0	Elongation factor Tu	27.45	26.08	11	11	16	43.4
F9UNV2	Bifunctional protein: transcription regulator sugar kinase, ROK family	27.16	29.38	9	9	9	33.7
F9UT64	Glycerol-3-phosphate cytidylyltransferase	27.04	41.79	10	10	14	15.8
Q88YM6	10 kDa chaperonin	26.17	62.77	5	5	9	10.3
F9UMU7	Lipoprotein, pheromone	26.06	31.20	8	8	8	42.6
F9UTV3	Multicopper oxidase	24.09	15.77	6	6	9	56.8
Q88XY7	30S ribosomal protein S10	23.78	47.06	6	6	9	11.7
F9UPE2	Transcription regulator, MarR family	23.33	35.00	6	6	8	15.6
F9UTI7	7,8-dihydroneopterin aldolase	22.66	34.43	4	4	9	13.8
F9UN59	Phosphocarrier protein Hpr	22.61	27.27	2	2	8	9.4
Q88YH4	Triosephosphate isomerase	22.06	31.35	7	7	8	27.0

F9UNJ5	Nucleotide-binding protein, histidine triad family [F9UNJ5_LACPL]	22.02	42.67	5	5	7	17.0
F9UMC8	Alkaline shock protein	21.99	36.43	6	6	7	15.8
F9UQB2	Cysteine desulfurase associated protein,DUF1831 family	21.16	64.60	8	8	9	12.6
F9UPK7	DNA-binding protein	20.76	35.16	4	4	6	9.6
F9UNU7	Transpeptidase (Penicillin binding protein 2B)	20.29	9.44	7	7	7	72.6
Q88VJ2	D-lactate dehydrogenase	19.97	24.10	8	8	8	37.2
F9USV1	Small heat shock protein	19.91	57.86	6	6	7	16.0
Q88WN3	50S ribosomal protein L27	19.57	39.13	4	4	7	10.1
F9UL15	Ribosome-associated heat shock protein	19.14	35.79	5	5	6	10.9
Q88XY2	30S ribosomal protein S19	18.79	45.05	5	5	8	10.3
F9UU72	Prophage P3 protein 2, CI-like repressor	18.75	41.27	6	6	6	13.8
F9UNV4	Rhodanese family protein	18.74	48.18	5	5	8	15.8
F9UU57	Uncharacterized protein	18.59	29.92	5	5	6	13.8
Q88XX7	30S ribosomal protein S17	18.42	32.58	5	5	8	10.3
Q88XY5	50S ribosomal protein L4	17.35	27.05	6	6	7	22.6
F9URR0	Flavodoxin	16.62	41.89	5	5	5	16.0
Q88T35	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2	16.59	31.74	5	5	7	26.1
F9UNT7	Transcription regulator, MarR family	16.49	26.05	3	3	6	13.7
Q88XX6	50S ribosomal protein L14	16.49	43.44	5	5	7	13.1
Q88XX0	50S ribosomal protein L18	16.43	30.51	5	5	7	13.0
F9USY7	Maltodextrin-binding protein	16.38	23.02	7	7	7	45.6
Q88WM9	Transcription antitermination protein NusB	16.31	35.25	6	6	7	15.7
F9UM75	Phosphohydrolase, MutT/nudix family	16.25	36.69	4	4	5	15.6
Q88YW8	50S ribosomal protein L10	16.20	25.75	4	4	5	17.9
F9UNI1	GTP cyclohydrolase-2	16.10	11.88	4	4	6	43.6
F9UTT2	Fructose-bisphosphate aldolase	15.95	23.00	6	6	6	30.9
F9UPT6	Uncharacterized protein	15.81	27.89	6	6	7	16.1
F9URF7	Uncharacterized protein	15.33	30.00	3	3	6	13.8
F9UPZ9	Nucleic-acid-binding protein implicated in transcription termination	14.93	36.36	5	5	9	11.2
F9UUA2	Uncharacterized protein	14.70	53.66	5	5	5	14.5
Q88XY6	50S ribosomal protein L3	14.57	33.01	5	5	5	22.7
Q890K2	30S ribosomal protein S6	14.46	50.51	5	5	6	11.4
Q88XU7	30S ribosomal protein S9	14.31	27.69	4	4	8	14.6
F9UPM3	Pyruvate kinase	14.07	11.95	6	6	6	62.8
Q88YI6	S-ribosylhomocysteine lyase	13.14	22.15	3	3	4	17.4
F9UQM5	Cell shape-determining protein MreC	13.10	22.06	5	5	5	30.1
F9US72	6-pyruvoyl-tetrahydropterin synthase family protein	12.88	31.15	4	4	5	14.0
Q88VD5	30S ribosomal protein S15	12.82	22.47	4	4	5	10.5
F9UN40	Glycerol-3-phosphate cytidylyltransferase	12.82	28.79	4	4	5	15.5
Q88YX0	50S ribosomal protein L11	12.39	26.95	5	5	6	14.8
Q88XZ6	Transcriptional regulator CtsR	12.31	23.23	4	4	4	17.6
F9UQC9	Transpeptidase, penicillin binding protein 2B	11.84	6.73	4	4	4	77.2
Q88XW9	30S ribosomal protein S5	11.54	25.30	5	5	6	17.3
F9USS0	Pyridoxamine 5'-phosphate oxidase family protein, FMN-binding	11.41	24.31	3	3	4	16.0
F9UMI2	Cold shock protein CspC	11.39	87.88	3	4	5	7.3
F9UUF6	FMN-binding protein	11.35	26.23	2	2	5	13.4

Q88VK8	Ribosome-binding factor A	11.21	30.77	6	6	6	13.1
F9UPF6	Uncharacterized protein	10.95	37.93	4	4	4	13.1
Q88W32	Probable manganese-dependent inorganic pyrophosphatase	10.87	17.48	4	4	4	33.6
Q88XX4	50S ribosomal protein L5	10.71	29.44	5	5	5	20.2
Q88XZ0	30S ribosomal protein S12	10.58	25.55	4	4	5	15.2
Q88RZ2	D-ribose pyranase	10.51	19.85	2	2	4	14.6
F9UQP4	Universal stress protein	10.40	24.38	3	3	4	17.5
F9UNC2	Extracellular protein, membrane-anchored	10.23	22.32	2	2	3	12.5
F9URG1	Ribonucleotide reductase protein NrdI CPL]	10.21	22.36	3	3	4	18.2
F9UT98	Serine-type D-Ala-D-Ala carboxypeptidase	10.17	10.14	4	4	4	46.9
Q6LWH2	Uncharacterized protein	10.09	24.14	4	4	4	13.0
F9UTM5	Small heat shock protein	10.09	36.05	4	4	4	16.7
Q6LWF2	Uncharacterized protein	10.05	21.77	4	4	4	14.0
F9UT53	Cystathionine beta-lyase / cystathionine gamma-lyase	9.96	13.91	5	5	5	40.8
Q88WA8	Asparagine--tRNA ligase 2	9.91	8.33	3	3	3	49.8
F9UL56	PTS system, mannose-specific EIIAcomponent	9.80	19.42	2	2	4	15.3
F9UNV8	Glutamine synthetase repressor, MerR family	9.77	37.30	4	4	4	14.5
Q88XU8	50S ribosomal protein L13	9.73	29.93	4	4	4	16.2
F9UPV5	Uncharacterized protein	9.71	34.38	4	4	5	11.7
F9UTM1	Uncharacterized protein	9.70	27.10	2	2	3	12.1
F9URL6	Transcription regulator, GntR family	9.39	27.64	3	3	4	14.1
F9UNS7	Two-component system response regulator	9.33	8.33	3	3	4	26.4
F9UM05	Oligopeptide ABC transporter, substrate binding protein	9.32	9.27	5	5	5	61.0
F9URE5	Universal stress protein	9.21	18.30	2	2	3	16.9
F9USJ7	Transcription regulator, MarR family	9.13	40.83	5	5	6	13.8
F9UTM0	Uncharacterized protein	9.03	39.52	5	5	5	14.4
F9UTD0	Uncharacterized protein	8.91	27.21	4	4	4	16.8
Q88Z97	Methionine--tRNA ligase	8.87	5.12	2	2	3	76.7
F9UQQ5	Glycine cleavage system, H protein	8.82	24.49	2	2	3	10.5
Q88XX1	50S ribosomal protein L6	8.81	18.54	3	3	3	19.3
Q88VM0	Chaperone protein DnaK	8.76	5.63	3	3	3	66.7
F9UMW7	Uncharacterized protein	8.72	23.29	2	2	3	16.2
F9UM38	Cell surface protein, YbbR-like family	8.62	11.69	2	2	2	34.6
P71478	Cold shock protein 1	8.62	50.00	3	4	4	7.3
F9UMV3	Transcription regulator, TetR family	8.53	13.61	2	2	3	21.6
Q88VC6	UPF0298 protein lp_2135	8.46	46.00	3	3	3	11.8
Q88VJ4	30S ribosomal protein S2	8.34	11.99	3	3	3	30.2
F9UNR4	RNA binding protein, YhbY family	8.25	38.46	3	3	3	11.7
F9URP8	PTS system, cellobiose-specific EIIA component	8.23	25.22	3	3	3	12.5
F9UQK6	Hypothetical membrane protein	8.17	2.74	2	2	3	114.8
F9UTR5	Acetyltransferase, GNAT family	8.11	28.57	4	4	4	17.1
Q890K0	30S ribosomal protein S18	8.08	38.46	4	4	4	9.1
F9UMC6	Hypothetical membrane protein	8.08	18.23	3	3	3	20.4
Q88X16	6,7-dimethyl-8-ribityllumazine synthase	7.85	12.58	3	3	3	16.7
F9URD6	Uncharacterized protein	7.77	21.48	2	2	2	16.0
F9UL94	Prophage P1 protein 8, phage transcription regulator, Cro/CI family	7.46	28.33	5	5	5	13.8
F9URC4	Transcription regulator, Cro/CI family	7.38	21.74	3	3	3	16.0

F9URX6	Universal stress protein	7.24	20.41	3	3	5	16.3
F9UNZ3	Serine/threonine protein kinase	7.18	4.15	2	2	2	74.5
Q88UU0	ATP synthase subunit delta	7.17	20.99	3	3	3	20.0
F9US78	Flavoprotein	7.17	19.15	2	2	2	15.1
F9UTF5	Zinc-dependent proteinase	7.15	12.07	2	2	2	25.2
Q88VQ3	D-aminoacyl-tRNA deacylase	6.95	19.59	2	2	2	16.0
F9UNH5	Nucleoside 2-deoxyribosyltransferase	6.95	25.34	3	3	3	16.6
F9UL76	Mini-ribonuclease 3	6.67	19.12	2	2	2	15.3
Q88XW7	50S ribosomal protein L15	6.67	19.58	2	2	2	15.3
F9UP44	Acetyl-CoA carboxylase, carboxyl transferase subunit alpha	6.59	10.16	2	2	2	27.6
F9UQF5	Uncharacterized protein	6.44	25.40	2	2	2	14.4
F9URS2	Extracellular protein	6.41	12.67	2	2	2	23.2
Q88X33	UPF0342 protein lp_1415	6.39	25.44	2	2	2	13.2
F9UP60	Adherence protein, chitin-binding domain	6.13	9.45	2	2	2	22.2
Q88YP3	Initiation-control protein YabA	6.09	23.48	2	2	3	13.6
P56512	L-lactate dehydrogenase 1	5.97	9.38	2	2	2	34.2
Q88YH5	Phosphoglycerate kinase	5.94	9.50	4	4	5	42.8
F9UN36	Transcription regulator, Cro/CI family	5.92	17.60	2	2	2	13.8
Q88VR5	30S ribosomal protein S21	5.86	41.94	3	3	4	7.6
F9UMI5	Transcriptional attenuator, cell envelope-related, LytR family	5.69	6.38	2	2	2	37.7
Q88WK0	Ribonuclease 3	5.64	9.09	2	2	2	26.6
Q88Z02	Aspartate 1-decarboxylase	5.52	18.46	2	2	2	14.4
Q6LWH4	Uncharacterized protein	5.51	23.64	2	2	2	13.0
F9UT92	Uncharacterized protein	5.18	11.11	2	2	3	16.6
Q88XX5	50S ribosomal protein L24	5.18	9.71	2	2	2	11.4
F9UNR7	Ribosomal silencing factor RsfS	5.16	24.58	2	2	2	13.0
F9UP36	Acyl carrier protein	5.12	24.39	2	2	2	9.3
Q88Z44	Holo-[acyl-carrier-protein] synthase	5.12	15.83	2	2	2	13.2
F9US19	Lipoprotein	5.08	12.74	2	2	3	17.3
F9UQY9	Prophage P2a protein 7 extracellular protein with lipoprotein anchor	4.91	23.42	2	2	2	12.0
F9UL53	Uncharacterized protein	4.87	25.98	4	4	4	14.8
Q88XY8	Elongation factor G	4.86	3.15	2	2	2	77.0
F9UT55	Methionine aminopeptidase	4.74	9.89	2	2	2	29.1
F9USC3	Nucleotide-binding protein, universal stress protein UspA family	4.67	25.87	3	3	3	15.7
F9UU77	Uncharacterized protein	4.57	21.15	3	3	3	17.6
F9UL89	Prophage P1 protein 2, mitogenic factor, cell surface lipoprotein	4.57	9.56	2	2	3	41.8
F9UNU5	Uncharacterized protein	4.56	11.00	2	2	2	11.5
F9UTM3	Uncharacterized protein	4.40	12.40	2	2	2	14.0
F9URL9	Nucleotide-binding protein, universal stress protein UspA family	4.39	14.10	2	2	2	17.1
Q88S55	Transcription regulator, Rrf2 family	4.25	20.57	2	2	2	15.6
F9URV6	Transcription regulator, MerR family	4.20	19.44	3	3	3	16.9
F9URP6	6-phospho-beta-glucosidase	4.08	5.63	2	2	2	54.7
F9URG4	Transcription regulator, MerR family	4.05	25.60	3	3	3	14.5
F9UR52	Lactoylglutathione lyase family protein	3.91	21.48	3	3	3	16.9
F9UP45	Enoyl-[acyl-carrier-protein] reductase [NADH]	3.67	6.35	2	2	2	26.9
F9UL47	PTS system, mannose-specific EIID component	3.67	6.56	2	2	3	33.6
Q88XY0	30S ribosomal protein S3	3.60	17.97	3	3	3	24.2
F9UMW3	Universal stress protein	3.12	14.38	2	2	2	15.8

Q88XW3	50S ribosomal protein L36	2.76	30.77	2	2	2	4.6
Q88VQ7	Histidine--tRNA ligase	2.64	5.16	2	2	2	47.9
F9UPK2	Uncharacterized protein	2.33	14.00	2	2	2	17.1
F9UL91	Prophage P1 protein 5, superinfection exclusion (Cell surface N-anchored)	2.29	8.91	3	3	4	37.9
F9ULD4	RNA polymerase (RNAP)-binding regulatory protein, arsenate reductase (ArsC) family, Spx subfamily	2.18	22.07	3	3	3	16.7
F9UN56	Transcription regulator, MarR family	2.14	17.05	2	2	2	14.4
Q88X97	Large-conductance mechanosensitive channel	2.10	13.49	2	2	3	14.0
Q88WG0	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta 2	1.86	6.76	2	2	2	30.9
F9UMY6	dTDP-4-dehydrorhamnose 3,5-epimerase	0.00	8.81	2	2	2	21.8

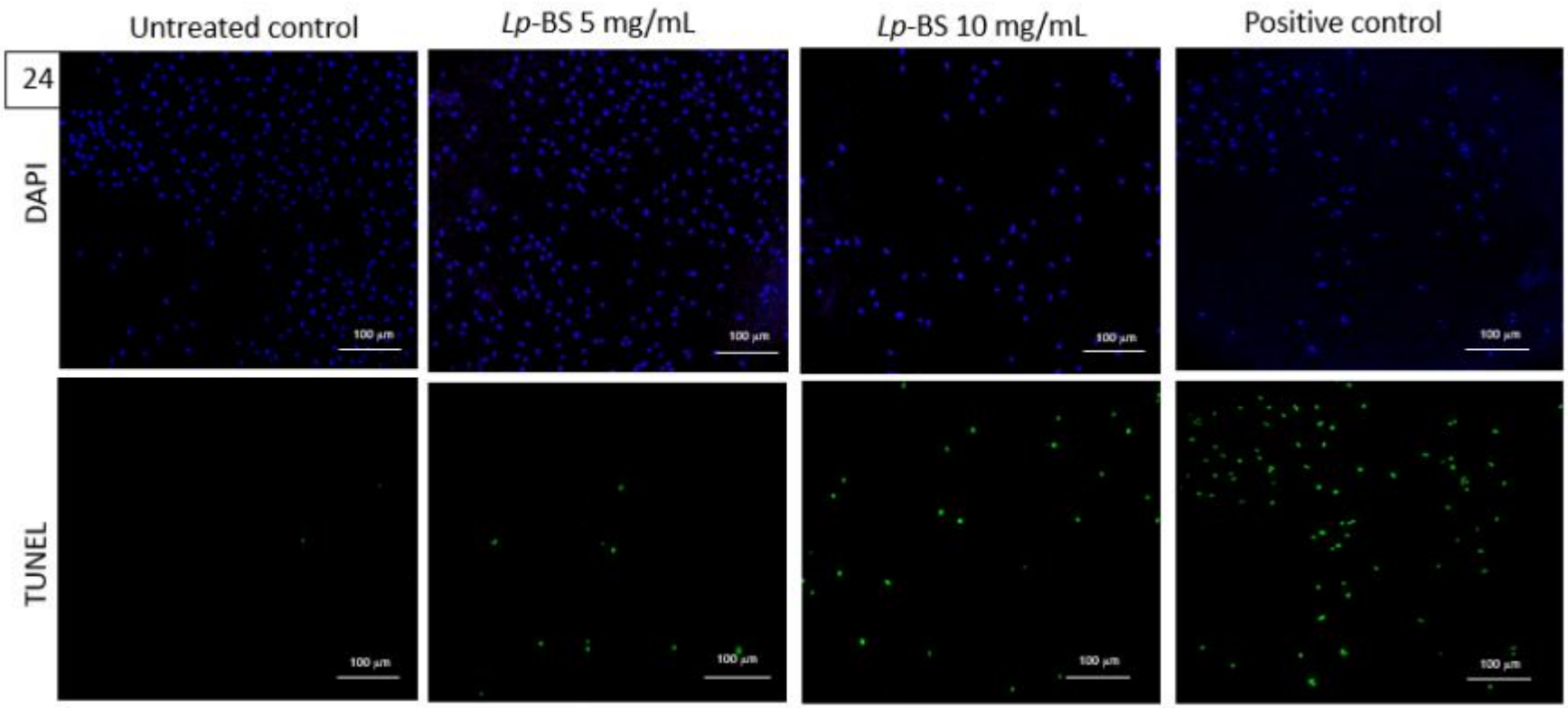
Band # 10

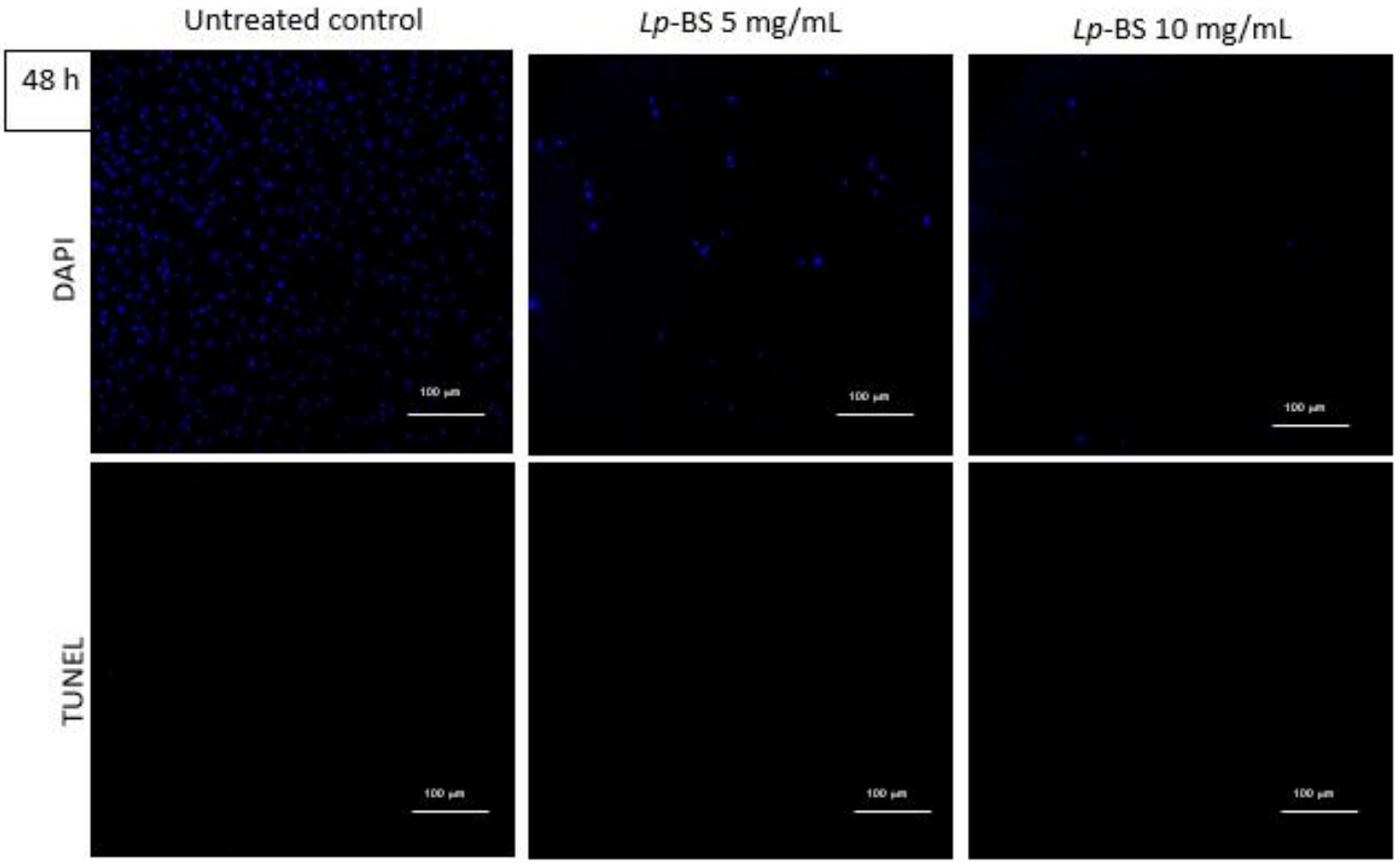
Accession	Description	Score	Coverage	# Unique Peptides	# Peptides	# PSMs	MW [kDa]
F9URG7	Glucose-6-phosphate 1-dehydrogenase	41.17	24.90	10	10	16	56.8
Q88W76	Formate--tetrahydrofolate ligase	31.94	14.34	5	5	9	57.9
F9UPL0	30S ribosomal protein S1	30.93	30.30	10	10	13	47.1
Q88Y74	GMP synthase [glutamine-hydrolyzing]	28.35	25.48	10	10	10	57.4
F9UQC4	Cell division protein FtsA	27.49	21.88	7	7	9	48.3
F9UPM3	Pyruvate kinase	27.13	16.04	7	7	9	62.8
Q88YH5	Phosphoglycerate kinase	26.04	28.75	9	9	9	42.8
F9UQC3	Cell division protein FtsZ	25.85	22.95	6	6	9	45.0
F9UMF3	Dipeptidase	24.31	15.38	6	6	9	52.3
F9UTT6	Dipeptidase	22.91	13.40	6	6	7	52.9
Q88YM5	60 kDa chaperonin	22.16	16.27	6	6	7	57.4
F9UM10	Glyceraldehyde 3-phosphate dehydrogenase	19.34	23.24	6	6	6	36.4
F9UT60	Trehalose-6-phosphate hydrolase	17.60	9.87	5	5	6	62.0
F9URU7	Poly(Glycerol-phosphate) alpha-glucosyltransferase [F9URU7_LACPL]	17.32	11.87	6	6	6	58.3
Q88UU1	ATP synthase subunit alpha	17.12	15.48	7	7	7	54.5
Q88Z28	Lysine--tRNA ligase	17.01	10.82	5	5	8	57.6
Q88UI4	Glucose-6-phosphate isomerase	16.34	6.89	3	3	6	49.8
Q88YY0	Glutamate--tRNA ligase	15.87	17.94	6	6	8	56.9
F9UM05	Oligopeptide ABC transporter, substrate binding protein	15.72	8.18	4	4	5	61.0
Q88UU3	ATP synthase subunit beta	15.21	12.42	4	4	4	50.8
Q88YX9	Cysteine--tRNA ligase	13.93	12.98	5	5	6	52.7
Q88YH3	Enolase 1	13.02	9.50	3	3	4	48.0
Q88VM0	Chaperone protein DnaK	12.46	9.81	4	4	4	66.7
F9UN83	Glycosyl transferase, group I	12.44	7.72	4	4	5	62.8
Q88YE7	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	11.98	7.93	4	4	4	65.4
F9URP6	6-phospho-beta-glucosidase	11.49	11.67	4	4	4	54.7
Q88Y23	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase	10.86	7.51	3	3	4	53.8
F9UNS5	6-phosphogluconate dehydrogenase, decarboxylating	10.71	7.11	3	3	4	52.9
P56512	L-lactate dehydrogenase 1	10.43	10.94	3	3	3	34.2
Q88YH4	Triosephosphate isomerase	10.28	16.67	3	3	3	27.0
Q88XP6	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	10.05	8.44	3	3	3	53.2

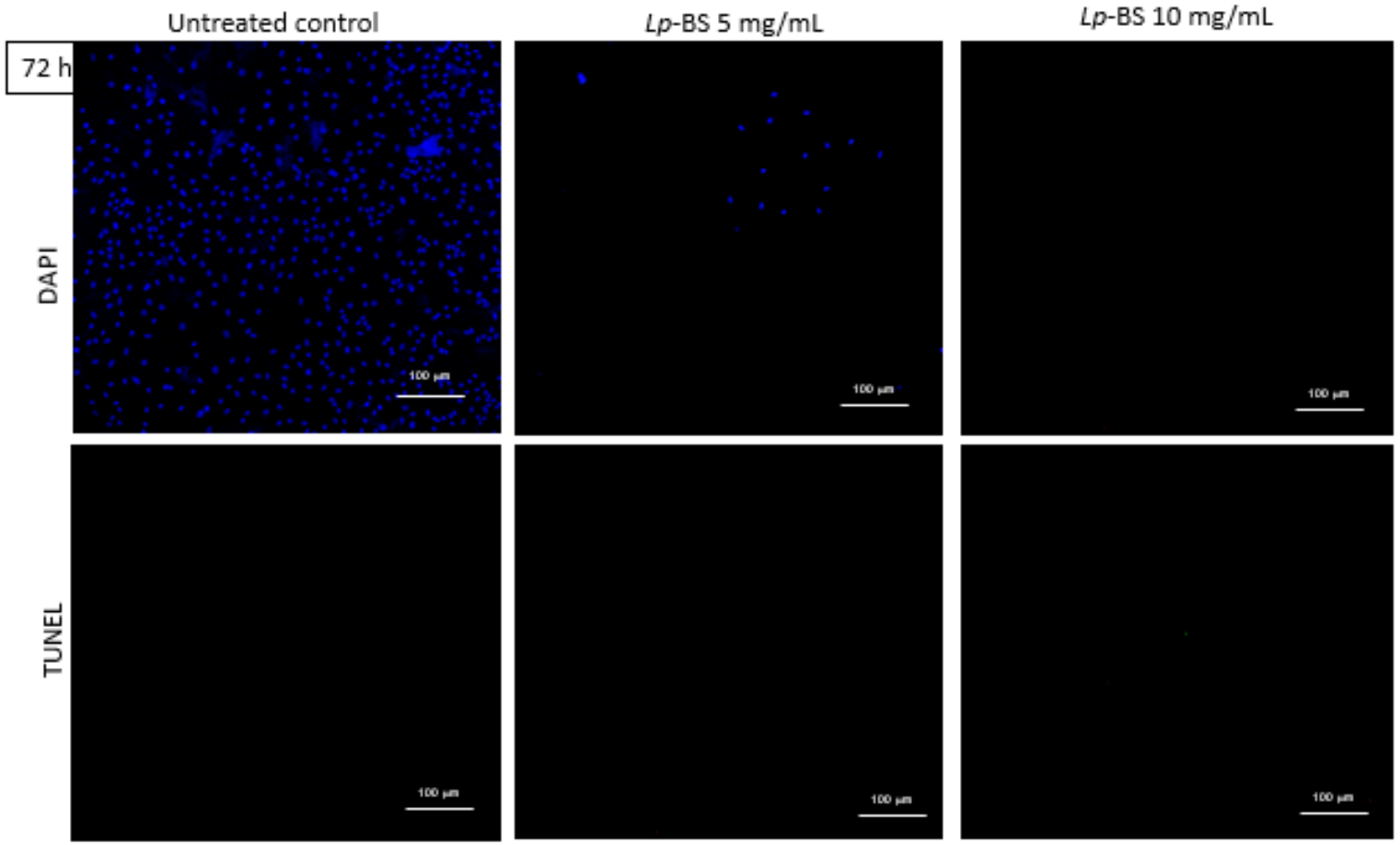
F9UQ90	Dihydrolipoyl dehydrogenase	9.94	11.06	4	4	4	49.9
F9UN93	Xaa-His dipeptidase	8.35	14.13	4	4	4	50.8
F9UTG0	Glutathione reductase	7.91	8.56	3	3	3	48.2
Q88VM6	D-alanine--D-alanyl carrier protein ligase	7.84	6.10	2	2	4	56.0
Q88VJ5	Elongation factor Ts	7.53	10.96	2	2	2	31.6
Q88VE0	Elongation factor Tu	7.46	12.15	4	4	4	43.4
F9UQ97	Ribonuclease J	7.36	6.09	3	3	3	61.8
F9UTT2	Fructose-bisphosphate aldolase	7.32	10.80	2	2	2	30.9
F9UTQ7	Extracellular transglycosylase	7.19	9.74	2	2	2	26.3
F9UT31	Dipeptidase	6.90	6.37	2	2	2	52.5
F9UT87	Cyclopropane-fatty-acyl-phospholipid synthase	6.89	6.30	2	2	3	45.3
Q88V80	UDP-N-acetylmuramoylalanine--D-glutamate ligase	6.59	6.10	2	2	2	50.1
Q88X53	Arginine--tRNA ligase	5.94	3.91	2	2	2	62.9
F9UNV2	Bifunctional protein: transcription regulator sugar kinase, ROK family	5.35	7.81	2	2	2	33.7
F9UQ85	Extracellular protein, cell-wall anchored	5.23	5.08	2	2	2	45.8
Q88VJ2	D-lactate dehydrogenase	4.74	12.95	4	4	4	37.2
F9UUI9	6-phospho-beta-glucosidase	4.59	4.38	2	2	2	54.5
F9UP39	3-oxoacyl-[acyl-carrier-protein] synthase 2	3.14	3.66	2	2	2	42.4
F9URU6	Poly(Glycerol-phosphate) alpha-glucosyltransferase	2.41	4.58	2	2	2	57.6
F9UN84	Poly(Glycerol-phosphate) alpha-glucosyltransferase	2.29	4.38	2	2	2	57.8
F9UN85	Poly(Glycerol-phosphate) alpha-glucosyltransferase	2.20	5.31	2	2	2	57.8
F9URM9	Threonine synthase	1.76	5.03	3	3	3	54.5

Appendix II: Apoptotic effect of *Lp*-BS and rhamnolipid, magnified photomicrographs.

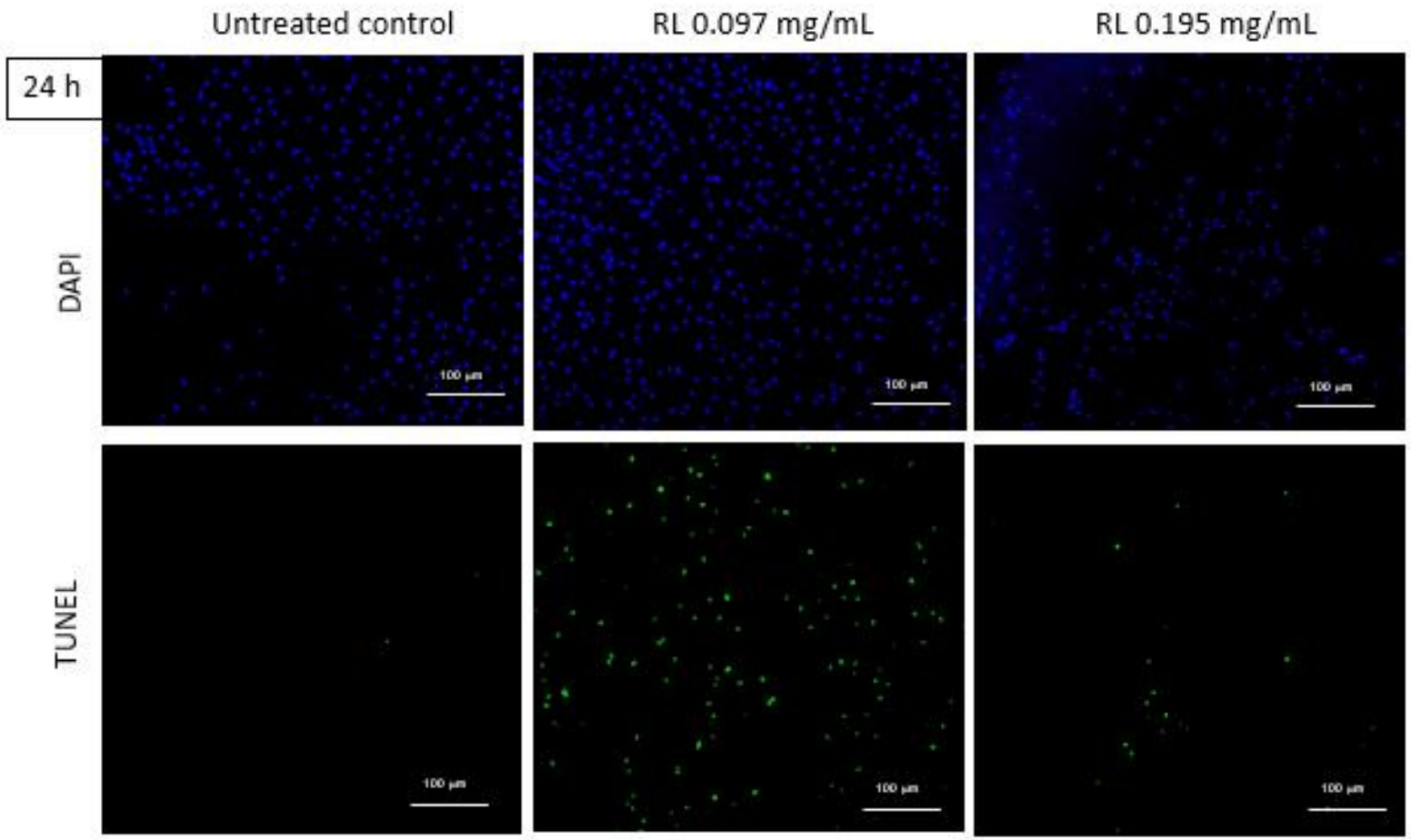
1- *Lp*-BS apoptotic effect

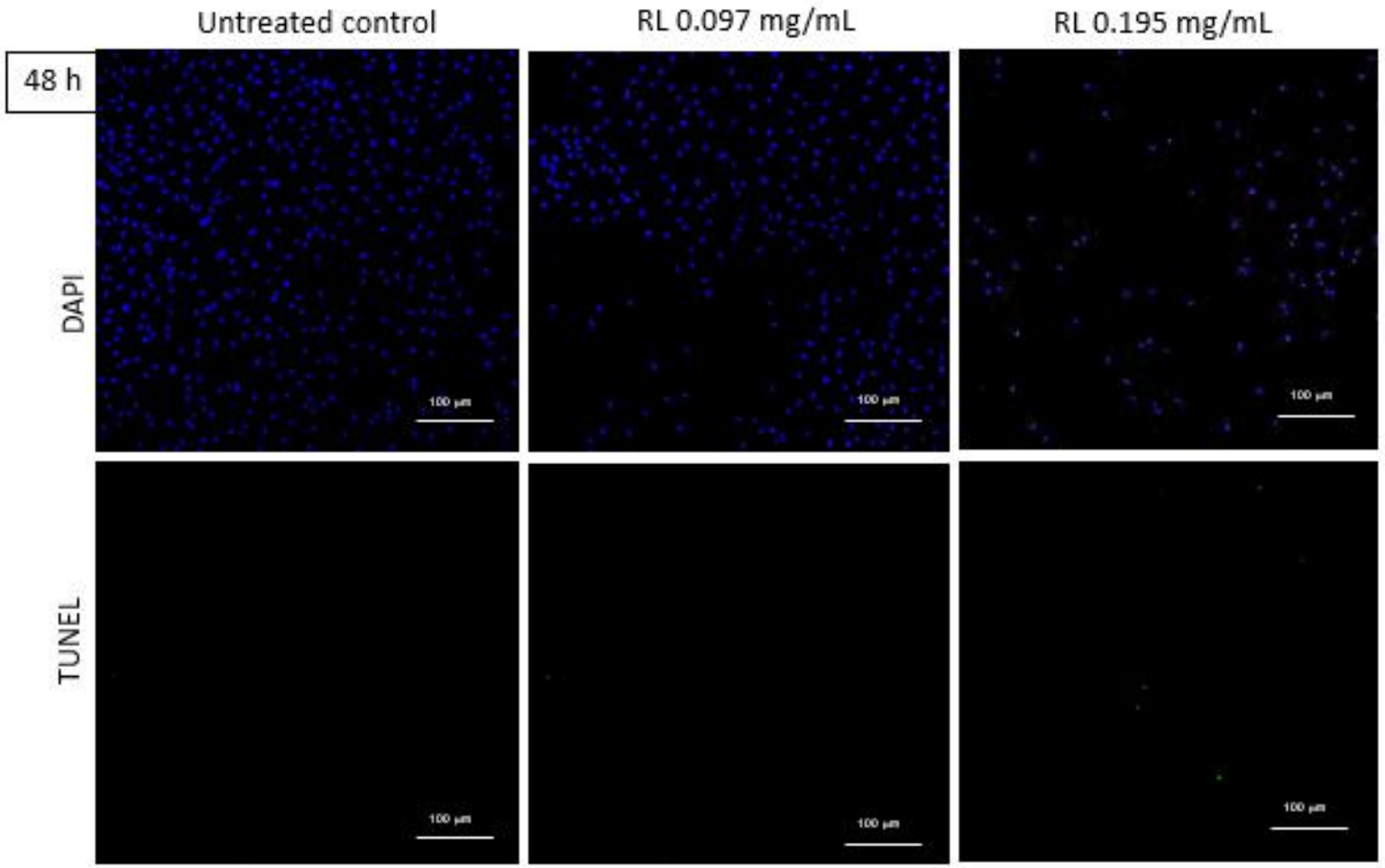


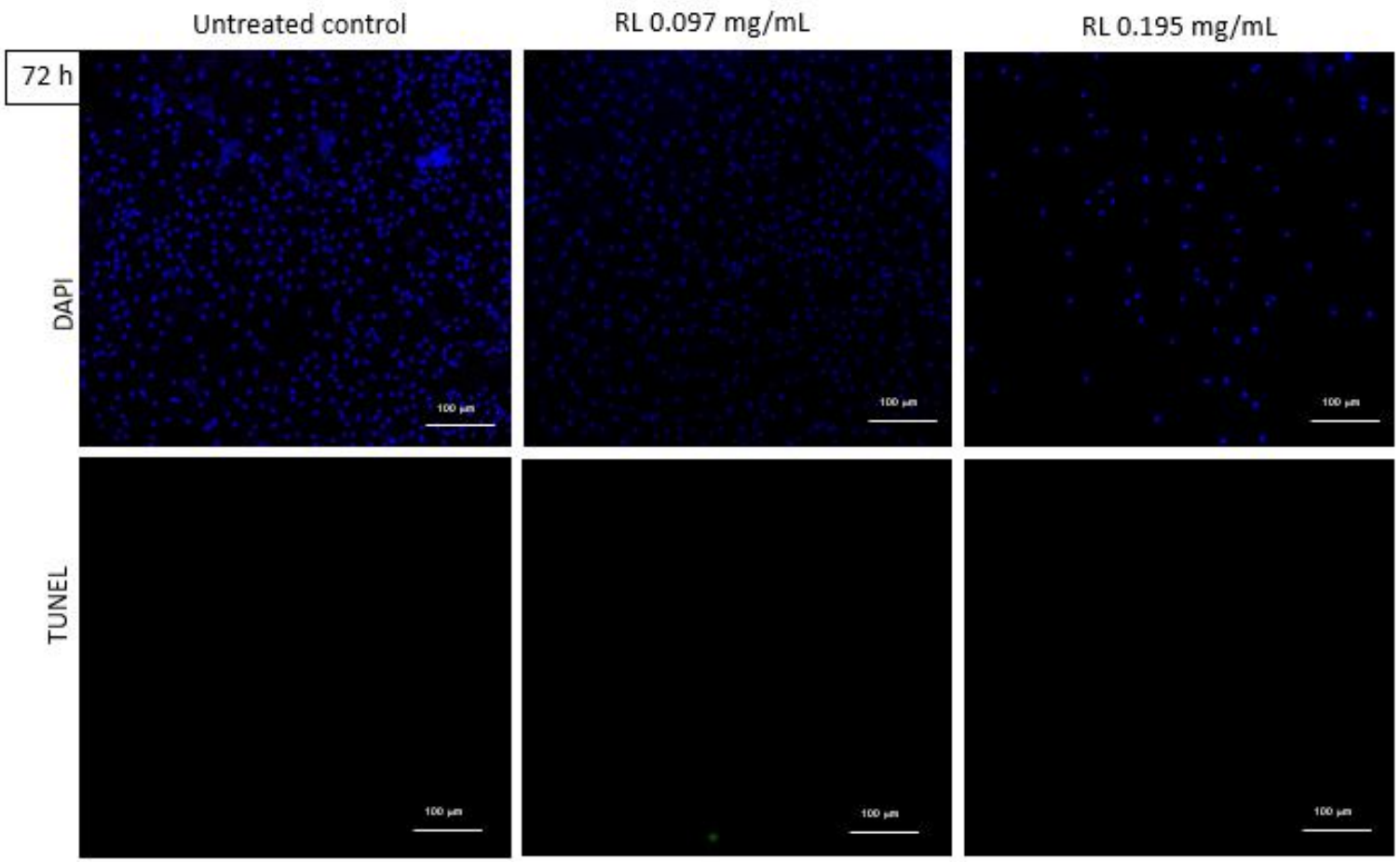

Untreated control
Lp-BS 5 mg/mL
Lp-BS 10 mg/mL
48 h
DAPI
TUNEL
100 µm


Untreated control
Lp-BS 5 mg/mL
Lp-BS 10 mg/mL
72 h
DAPI
TUNEL
100 µm

2- Rhamnolipid apoptotic effect




Untreated control
RL 0.097 mg/mL
RL 0.195 mg/mL
48 h
DAPI
TUNEL
100 µm


Untreated control
RL 0.097 mg/mL
RL 0.195 mg/mL
72 h
DAPI
TUNEL
100 µm