Probiotic Interaction with Common Skin Pathogens: Understanding the Impact of Probiotics on Pathogen Function and Metabolism within the Host

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By

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1 List of Abbreviations

AAD	Antibiotic-Associated Diarrhoea
AD	Atopic Dermatitis
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BCCM/LMG	Belgian Co-ordinated Collections of Microorganisms
BLAST	Basic Local Alignment Search Tool
BR	Broad-Range
CA-SSTIs	Community-Acquired Skin and Soft Tissue Infections
CFSs	Cell Free Supernatants
CFUs	Colony Forming Units
CP	Capsular Polysaccharides
Cul	Cultech
DMSO	Dimethyl Sulfoxide
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen aka, German Collection of Microorganisms and Cell Cultures
EAggEC	Enteroaggregative *Escherichia coli*
ECM	Extracellular Matrix
EFSA	European Food Safety Authority
Emps	Extracellular Matrix-Binding Proteins
ETA	Exfoliative Toxin A
ETB	Exfoliative Toxin B
FAB	Fastidious Anaerobe Broth
FAO	Food and Agricultural Organization
FDA	Food and Drug Agency
Fn	Fibronectin
FnBPs	Fibronectin-Binding Proteins
GAS	Group A Streptococci
GCGS	Groups C and G streptococci
GGS	Group G Streptococci

GI	Gastrointestinal
GRAS	Generally Regarded As Safe
HA	Hyaluronic Acid
HA-SSTIs	Hospital-Acquired Skin and Soft Tissue Infections
HL	Hyaluronate Lyase
HT	Heat-Treated
HTST	High Temperature Short Time
IBD	Irritable Bowel Disease
IBS	Irritable Bowel Syndrome
IgG	Immunoglobulin G
IOEB	Institute d'Oenologie de Bordeaux
ISAPP	International Scientific Association for Probiotics and Prebiotics
JGI	Joint Genome Institute
LAB	Lactic Acid Bacteria
LABGC	Lactic Acid Bacteria Genome Consortium
LD	Lethal Dose
LukD-E	Leukocidin D-E
MHA	Muller-Hinton Agar
MLST	Multilocus Sequence Typing
MRS	DeMan Rogosa and Sharp
MRSA	Methicillin Resistant *S. aureus*
MSCRAMMs	Microbial Surface Components Recognizing Adhesive Matrix Molecules
MSSA	Methicillin Sensitive *S. aureus*
NCBI	National Center for Biotechnology Information
NCIMB	National Collection of Industrial Food and Marine Bacteria
NCTC	National Collection of Type Cultures
NGS	Next Generation Sequencing
OTUs	Operational Taxonomic Units
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHW	Public Health Wales

PLC	Phospholipase C
PTSAgs	Pyrogenic Toxin Superantigens
PVL	Panton-Valentine Leucocidin
QPS	Qualified Presumption of Safety
RDP	Ribosomal Database Project
SDSD	*S. dysgalactiae* subspecies *dysgalactiae*
SDSE	*S. dysgalactiae* subspecies *equisimilis*
SERAMs	Secretable Expanded Repertoire Adhesive Molecules
SOP	Standard Operating Procedure
SSS	Scalded Skin Syndrome
SSTIs	Skin and soft tissue infections
TAG	Triacylglycerols
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth
TSS	Toxic Shock Syndrome
TSST-1	Toxic Shock Syndrome Toxin-1
UTI	Urogenital Tract Infections
VRE	Vancomycin-Resistant Enterococci
WBCs	White Blood Cells
WGS	Whole Genome Sequencing
WHO	World Health Organization

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3 Summary

Introduction: Skin and soft tissue infections (SSTIs) are considered a major public health problem in many countries. Pathogenic bacteria have evolved complex mechanisms for infection and colonization of numerous host cells. Despite the presence of various antibiotics for the treatment of infectious diseases, their random, inappropriate and excessive use has resulted in increasing incidence of several side effects and the development of antimicrobial resistance. Consequently, there is an urgent need to develop new and safe treatments for infections, especially those of the skin. Different new therapeutic approaches are currently being increased such as bacteriotherapy using probiotics. Dairy and non-dairy fermented foods have been used in traditional diets worldwide and are still to be broadly consumed, since they are considered as rich sources of probiotics, especially lactic acid bacteria. From commencement to cure, the progress and severity of infectious diseases are continuously affected by the interaction between the pathogen and the host. Therefore, investigations performed both *in vitro* and *in vivo* are important when determining the health- enhancing properties of probiotics. The greater wax moth larvae *Galleria mellonella* is a well-accepted model of infection by scientists, as an *in vivo* model for host-pathogen interactions due to several advantages, such as its low cost and no required ethical rights to be used in the experiments *in vivo*.

Aim: This project aimed to investigate the antagonistic activity of *Lactobacillus* type strains and food isolates against the major causative agents of skin infections *in vitro.* It also aimed to explore the therapeutic potency of food-derived *Lactobacillus* species against skin pathogens, using both the injection method inside the *Galleria mellonella* larvae model, and the topical application on the larvae as a novel technique for infections and treatment which can assist in decreasing the occurrence of skin infections.

Results: A total of twenty *Lactobacillus* species, including ten type strains obtained from different culture collections and ten food isolates derived from fermented yogurt and olives, all revealed significant inhibitory activity against the three skin pathogens used in this study; *Staphylococcus aureus*, *Streptococcus pyogenes* and *Streptococcus dysgalactiae* subsp. *equisimilis.* Although the antagonistic effect was observed after the first day of incubation under both aerobic and anaerobic incubation conditions, the maximum antibacterial effect was detected following three days of anaerobic incubation. Furthermore, food-isolated *Lactobacillus* presented a higher inhibitory effectiveness than the type strains against the pathogens

particularly under the anaerobic conditions. Characterisation of bacterial genomes by whole genome sequence (WGS) analysis showed that the sequenced genomes of food lactobacilli had a number of genes encoding for antimicrobial substances, in addition to other genes responsible for probiotic advantages. Multiple genes encoding for virulence factors were also detected in the pathogenic sequenced genomes. Diversity of the bacterial community of food samples by next generation sequencing (NGS) of 16S rRNA gene amplicons revealed that *Lactobacillus delbrueckii* isolated from yogurt by the culture-dependent procedure was found in low abundance as *Lb. delbrueckii* subsp. *indicus* using the culture-independent method. Despite the isolation of *Lactobacillus plantarum* from olives using culturing technique, no signal was observed for this species by NGS.

Injection of *Lb. delbrueckii* and *Lb. plantarum* isolated from fermented foods in *G. mellonella* larvae presented a significant therapeutic activity of both species against pathogenic infections in a dose-dependent matter. Both the injected doses, 10^2 CFU/larva and 10^4 CFU/larva of *Lb. delbrueckii* washed cells, were effective in reducing *S. aureus* and *S. pyogenes* infections. Whereas the low injected dose of this species had more activity than the higher one to decrease the infection of *S. dysgalactiae* subsp. *equisimilis* in the larvae. Regarding *Lb. plantarum*, the doses of 10^3 CFU/larva and 10^4 CFU/larva injected in the larvae were both useful in reducing *S. aureus* infection, with more efficiency for the low dose. However, both doses had no therapeutic effect against *S. pyogenes* infection. The novel topical application technique of *S. pyogenes* cells on the dorsum of larvae followed by the application of *Lb. plantarum* washed cells and undiluted cell free supernatant (CFS) resulted in lower death rates of the treated larvae in comparison to the control group. Nevertheless, bacterial cells of *Lb. plantarum* revealed more activity than the CFS of the same species.

Conclusions: The significant antagonistic effect *in vitro* and the significant therapeutic potency of food *Lactobacillus* species against skin pathogens, indicate that food-derived *Lactobacillus* isolates included in this study could act as potential candidates with promising probiotic advantages. This research delivers the basis for further investigation on the treatment of skin infections with food lactobacilli. Moreover, these food-based *Lactobacillus* species could be introduced as topical formulations to decrease the symptoms of skin infections or even in the context of skin infection treatments.

Chapter One

General Introduction

1 General Introduction

1.1 Skin Microbiome

The skin is an ecosystem which frequently interacts with the external environment and is colonized with a number of different microorganisms including bacteria, fungi and viruses. All these together comprise the skin microbiome. However, bacteria are the major members of the microbiota that colonize the skin. These microbial groups are associated with human health and disease (Leyden *et al.* 1987; Chiller *et al.* 2001; Grice and Segre 2011; Kong 2011). Human skin is one of the first lines that protects the body against microbial invasion as it acts as a physical barrier by secreting fatty acids, sebaceous fluid and low pH to prevent growth of pathogens. Additionally, skin microbiota can also deter the colonization of other pathogenic microbes (McAdam and Sharpe 2005). The microbiota colonized the skin can be divided into two halves at the waistline. The typical organisms distributed on the skin above the waist are often Gram-positive species such as *Staphylococcus epidermidis*, *Corynebacterium* species, *Staphylococcus aureus* and *Streptococcus pyogenes*. Other Gram-positive and Gram-negative species usually colonized the skin below the waist such as *Enterobacteriaceae* and *Enterococcus* species (Hussan and Hunter 2020).

The main function of the skin is to assist as a defensive physical barrier that protects our bodies from possible attack by external organisms and toxic materials (Chiller *et al.* 2001). Microbes inhabiting the skin vary across different parts of the cutaneous surface composing on average 1.8 m^2 of adult human skin (Grice *et al.* 2009). The skin microbiota is classified by researchers as two groups: resident and transient. Resident microbes which are normally existing in and on the skin and can re-generate themselves after disruption. These microorganisms are usually regarded as commensal, they may be beneficial for the host. On the other hand, transient microbes that do not constantly establish themselves on the skin surface but they emerge from the environment and endure for hours to days (Otto 2009). Under normal circumstances, both groups are not pathogenic. However, resident and/or transient bacterial populations can cause infection after their colonization and proliferation if any disturbance occurs. Damaged skin barrier function, imperfect immune response and defective normal resident microbiota are all different kinds of disturbance which resulted in causing infection. For example, the skin commensal bacterium *S. epidermidis* can be an opportunistic pathogen in immunocompromised hosts (Otto 2009). Moreover, in asymptomatic carriers, *S. aureus* which is also an opportunistic pathogen, can be a resident bacterium.

Human skin layers mainly comprise of an upper stratified, cellular epidermis and a fundamental dermis of connective tissue (Figure 1.1). Underneath the dermis, is the fat layer of the skin which is located in the subcutaneous layer of tissue named as hypodermis. There is a variation of the fatty layer's thickness among individuals depending on the size and number of fat cells. A layer of striated muscles separates the hypodermis from the rest of the body (Breathnach 1971; Montagna *et al.* 2012). The epidermis which is the outer layer of the skin is considered as a physical barrier that is difficult to penetrate by microorganisms and toxins while it maintains nutrients and moisture in the body (Madison 2003; Segre 2006; Proksch *et al.* 2008). The upper layer of the epidermis, the stratum corneum, is consisted of enucleated keratinocytes that are known as squames. The skin is a self-renewing organ and the squamous cells and adherent bacteria continuously shed from the surface of the skin as a result of terminal differentiation (Fuchs and Raghavan 2002; Elias 2005; Segre 2006). Though the existence of the microbial populations on the skin would be resisted by these obstacles, these microbes usually cover the superficial layer and extend down into the skin appendages and glands (Grice *et al.* 2008). In general, dehydrated, cool healthy skin is an unfavourable environment for microbial growth. There are several factors that affect the composition of skin microbial communities. Some of these factors are fundamental like age, sex, immunity and genetics all have an effect on the skin microbial composition. External and environmental factors, such as hygiene, nutrition, occupation, geographic location and climate may also influence the microbial communities of the skin (Kong and Segre 2012; Hussan and Hunter 2020).

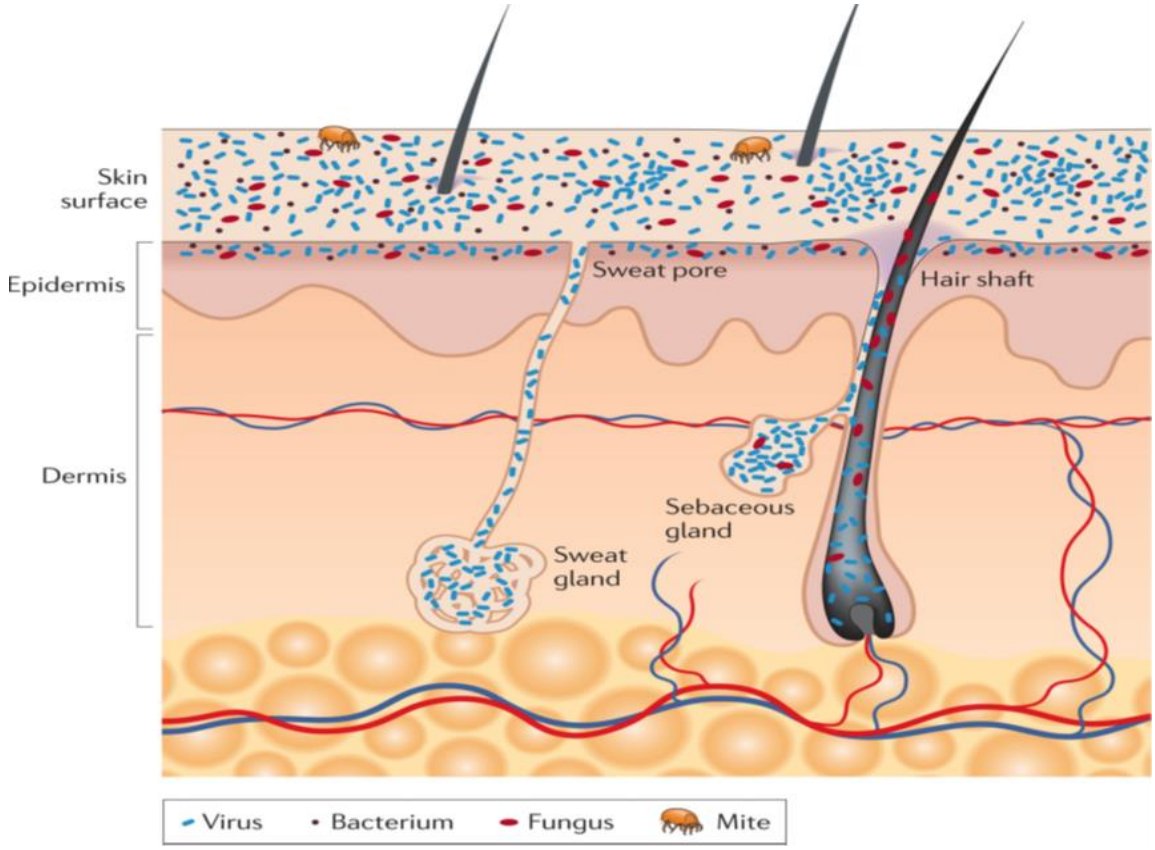

Figure 1.1 Human skin structure. Microbial populations (viruses, bacteria and fungi) and mites exist on the skin surface and are also deeply spread into the skin appendages (hair follicles, sweat glands and sebaceous glands). Image adapted from Grice and Segre (2011)

1.2 Skin Infections

Skin and soft tissue infections (SSTIs) are caused as a result of microbial invasion of the skin layers and underlying soft tissues. These infections have different etiological agents and severity. They vary from mild infections, like pyoderma, to severe infections, like necrotizing fasciitis (Ki and Rotstein 2008). Different skin layers (i.e., epidermis, dermis, subcutaneous and adipose tissue, and muscle fascia) are primarily colonized by bacteria in low numbers. When a disruption occurs in the integumentary barrier, the bacteria increase in number on the injury site followed by the invasion of these colonized bacteria and development of an SSTI (Ki and Rotstein 2008). There are four steps of SSTI development; adherence of bacteria to the epithelial cells of the host, bacterial tissue invasion with avoidance of host defences, elaboration of toxins and inducing the immune response of the host (Figure1.2) (McAdam and Sharpe 2005). Special proteins conferring these properties are encoded by virulence genes in several pathogenic bacteria. These bacteria can penetrate the skin barrier in different ways. The most frequent means

is through a disruption in the barrier (Figure 1.3). The entry of normal skin microbiota and indigenous microbiota can cause infections by several common mechanisms such as scratches, laceration and bite wounds, burns, surgery, instrument injuries (e.g., needles), wounds (e.g., chicken pox or ulcer) and previous skin conditions. Other means of skin penetration include water entry into skin pores (e.g., hot-tub folliculitis) and contagious transmission from adjacent infected body sites (e.g., osteomyelitis) (Eron *et al.* 2003; Stevens *et al.* 2005). The diagnostic symptoms of SSTIs are oedema, erythema, warmth and pain. The affected area may also lose their function (e.g. hands and legs) in the case of serious infections (Swartz 2004).

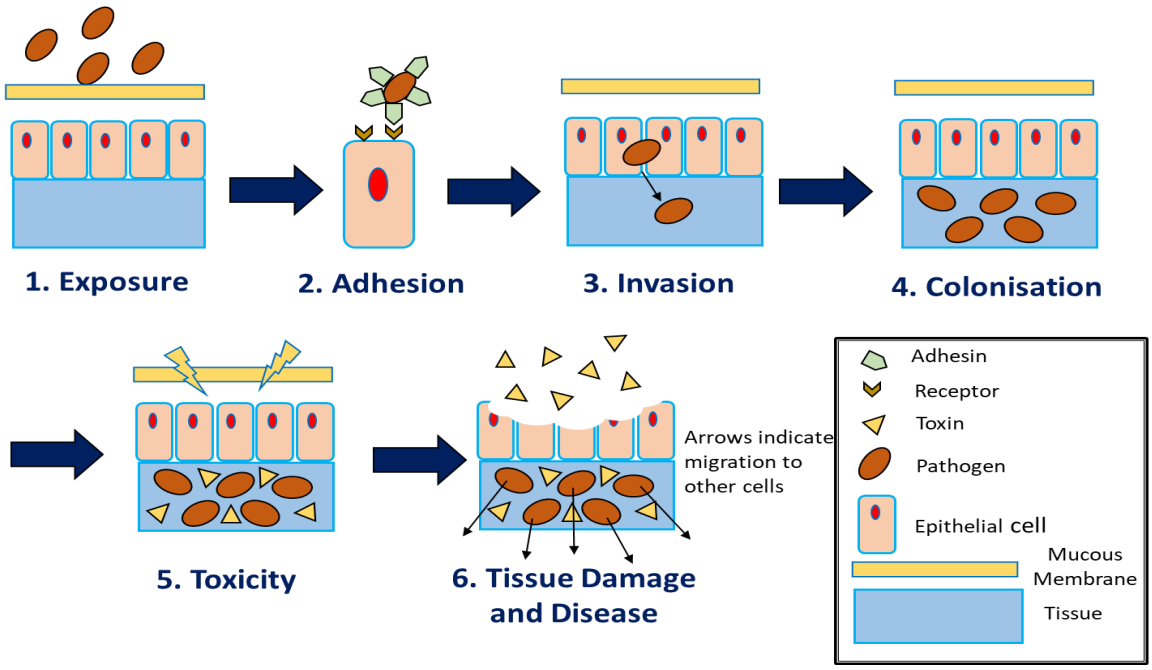

Figure 1.2 Development of skin and soft tissue infections. Pathogenic infection involves several steps starting upon the exposure to the pathogen. Skin and mucus membranes protect the body from pathogenic invasion. When a skin disruption occurs, the body becomes more susceptible to the infection. Pathogen passes through the mucous membrane and attaches to epithelial cells. This adherence allows the colonisation of pathogenic cells, which subsequently enables spread of reproduced cells into other tissues. At this step, the pathogen produces toxins which later result in tissue damage and disease (Image created using information from (Wikimedia_Commons_Contributors 2016).

Microorganisms that cause SSTIs are frequently normal microbiota of the host which are transferred either from their skin surface environment or penetrate from an instrument injury. Further, aetiological agents differ between community-acquired (CA) and hospital-acquired (HA) infections. Rennie and colleagues observed that more resistant bacteria were highly present in HA-SSTIs in North America (Rennie *et al.* 2003). These included *S. aureus* (45.9% of cases; methicillin resistant comprised 40% of all cases), *Pseudomonas aeruginosa* (10.8%),

Enterococcus species (8.2%). This contrasted with beta-haemolytic streptococci (2.3%) which were the major causatives of CA-SSTIs (Rennie *et al.* 2003). Methicillin resistant *S. aureus* (MRSA) has also increased in CA-SSTIs (Eady and Cove 2003; Frazee *et al.* 2005; King *et al.* 2006). This isolate was correlated with the Panton-Valentine leucocidin (PVL) virulence factor, and the genes encoding for PVL have been mainly described among CA-MRSA (Moroney *et al.* 2007). SSTIs infections are a major source of morbidity and mortality in hospitalized patients and their incidence is approximately 7% to 10% (Emori and Gaynes 1993; Vinh and Embil 2005). SSTIs are also very common in the emergency care units (Eron *et al.* 2003). Cutaneous infections are also caused by other bacteria, these include members of the *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and enterococci (Emori and Gaynes 1993).

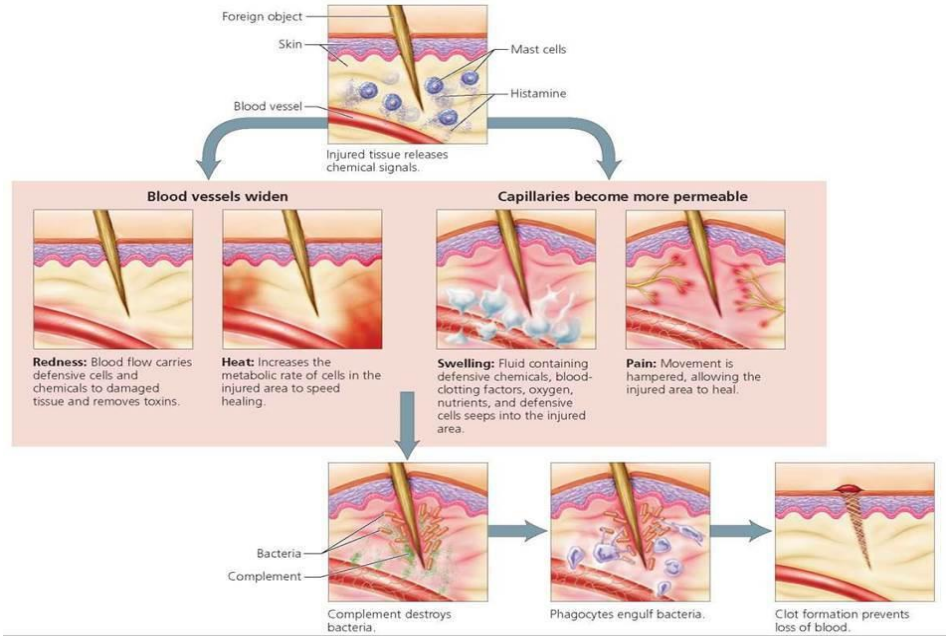

Figure 1.3 Skin defence mechanism in response to tissue damage and microbial invasion to protect the body from foreign pathogens and to clean the injured site from dead cells. The inflammation response includes four indications; redness, heat, swelling and pain. Image adapted from https://schoolbag.info/biology/humans/17.html.

Depending on the depth of infection and skin layers involved, SSTIs are divided into several types. These infections including the infected skin layer and the causative agent are described as follows: impetigo is a superficial bacterial infection. The development of this infection can occur either through direct attack of normal skin (primary) or infections of injured skin sites (secondary). It is extremely contagious and children are commonly infected. There are two types: non-bullous or crusted impetigo which is characterised by yellow crusting lesions usually on the face and extremities. Bullous impetigo is the second type; this infection is usually

caused by *S. aureus*. It appears as bullae (plural of bulla) that forms a brown crust after rupturing (Sukumaran and Senanayake 2016), A bulla is a lesion or a sac filled with trapped fluid beneath a thin skin layer. Boils and carbuncles are the infections of the hair follicles and subcutaneous tissue. They are painful and tender lesions (Sukumaran and Senanayake 2016).

Folliculitis is another skin condition that affects the hairy moist areas of the skin. The main cause of these is *S. aureus*. However, in some cases when an individual is exposed to spas and hot tubs, *Pseudomonas aeruginosa* can be the causative agent of folliculitis (Sukumaran and Senanayake 2016). Cellulitis and erysipelas infections are both exhibit as expanding areas of skin erythema and warmness. Some patients might also present with fever and systemic toxicity. Cellulitis expands deeply into the dermis and subcutaneous tissue. It normally affects the lower limbs and mostly one-sided. There are other serious types of cellulitis such as when the eye and eyelids become the foci of infection. This infection is commonly caused by either *S. aureus* or beta-haemolytic streptococci (groups A, B, C or G) (Sukumaran and Senanayake 2016). Erysipelas comprises the upper dermis and superficial lymphatics. The infected skin is clearly differentiated with elevated skin lesions. The face is the typical body site affected by infection however, it can also include other parts like the lower limb. *S. pyogenes* (group A Streptococcus) is a common cause of erysipelas (Sukumaran and Senanayake 2016).

One of skin infections is necrotising fasciitis. It is considered as a medical emergency which promptly requires a surgical procedure and intravenous antibiotics to treat. Several pathogens can cause these infections including Gram negatives, *Clostridium*, and *S. pyogenes*. Necrosis of soft tissues and muscles often accompany this necrotising infection. Thickening and redness of the affected area is accompanied by pain at the primary stages of infection. A skin colour change to blue or purple can happen as the infection develops and finally erupts to form bullae and gangrene (Sukumaran and Senanayake 2016).

1.3 Skin Infections Associated with *Staphylococcus aureus*

Staphylococcal infections are usually differentiated as pustules, furuncles or abscesses since a fibrin wall is formed in the surrounding margins of the infected area. Atopic dermatitis (AD, commonly known as eczema and contact dermatitis), is a regressive disorder that infects ~15% of United States children and ~2% of adults. The common causative agent of this disorder is *S. aureus* which mainly infects AD lesions. During the past three decades, the incidence of this disorder has increased in developed countries (Hanifin 2009). Environmental changes in

these countries may modify the gene-environment interaction on the skin surface which leads to the increase of the disease occurrence. These environmental differences include, an expansion in sanitary lifestyles, increased antibiotics use, decreased childhood infections and a decline in parasitic diseases (Hanifin 2009).

Some of the most hazardous skin infections are caused by Methicillin-resistant *S. aureus* isolates, especially when this bacterium excessively grows on human skin and mucous membranes (Klevens *et al.* 2007). Exotoxins produced by this bacterium can cause diseases such as, bullous impetigo, scalded skin syndrome and toxic shock syndrome (TSS) (Kollef *et al.* 2006). Hugo-Persson and Norlin (1987) mentioned that erysipelas is sometimes caused by *S. aureus* and cellulitis may be caused by a combination of both streptococci and *S. aureus*. In contrast bullous impetigo, a typical staphylococcal disease may possibly be caused by beta-haemolytic streptococci group A (Helsing and Gaustad 1992). *S. aureus* can also be the causative agent of dangerous illnesses like pneumonia, meningitis, osteomyelitis endocarditis, sepsis and bacteremia (Shafighi *et al.* 2012).

The ability of *S. aureus* to cause disease in the infected tissue of mammalian hosts is a result of the numerous virulence factors, such as protein A, coagulase and different membrane damaging toxins, four haemolysins (alpha-, beta-, gamma-, and delta- haemolysin) and leucocidin (Shana MOC 2009). Furthermore, there is a family of related pyrogenic toxins, namely staphylococcal enterotoxins, toxic shock syndrome toxin, and exfoliative toxins (Salasia *et al.* 2004).

1.4 Skin Infections Associated with *Streptococcus pyogenes*

Streptococcus pyogenes, is referred to Group A Streptococcus (GAS) since it belongs to Lancefield group A antigen group. It is an important species of Gram-positive bacterial pathogens. Species of this group usually inhabit the throat or skin and can be the main cause of a number of suppurative and non-suppurative infections (Wannamaker 1970). These bacteria are considered the most common cause of bacterial pharyngitis. Furthermore, scarlet fever and impetigo are also caused by group A streptococcus. Wannamaker (1970) showed that this group of bacteria has various serotypes, with some strongly associated with throat infections and others are frequently related to impetigo (Wannamaker 1970; Bisno 1995). Furthermore, this bacterial group is also responsible for streptococcal toxic shock syndrome. These bacteria are often known as the “flesh-eating” since they invade the skin and soft tissue, leading to the destruction of

infected tissues or limbs in severe cases. Severe infections with this bacterium leads to a high mortality ranging from 10% - 30% (Cunningham 2000). The initial step of *S. pyogenes* infections is bacterial adhesion to human epithelial cells of the oral and nasal cavities, and the skin (Sela *et al.* 1993). Hidalgo-Grass and co-workers also showed that a rapid spread of *S. pyogenes* to different organs usually occurs after the first invasion of the skin tissue. Escape of the bacterial cells from human immune system allows the initiation of systemic and severe infections (Hidalgo-Grass *et al.* 2004). It has been stated by McCornick and colleagues that *Streptococcus pyogenes,* which is one of the common skin pathogens, also produces staphylococcal pyrogenic toxins (McCornick *et al.* 2001).

1.5 Skin Infections Associated with *Streptococcus dysgalactiae* subsp. *equisimilis*

S. dyscalactiae subsp. *equisimilis,* designated as Groups C and G streptococci (GCGS) since it is classified within Lancefield groups C and G antigens. These bacteria which include multiple species with variable haemolytic features, biochemical reactions, and clinical infections that occur in both humans and animals. Over the last few decades, the classification of GCGS has been continuously developing. Members of these bacterial groups which cause a large number of human infections include either *S. constellatus* and *S. intermedius* (formerly known as the *S. milleri* group) or by *S. dysgalactiae* subspecies *equisimilis* (SDSE). Colonies of this bacterium grown on Sheep blood agar plates are large (>5mm) and typically beta-haemolytic, with the same culture characteristics as *S. pyogenes* (Facklam 2002; Broyles *et al.* 2009; Chochua *et al.* 2017). Beta-haemolytic streptococci have hyaluronidase, an enzyme that enables the bacteria to cause infections with a tendency to spread quickly through the connective tissue, causing cellulitis and erysipelas. Depending on the host, Vandamme *et al.* (1996) proposed two subspecies of *S. dysgalactiae,* including *S. dysgalactiae* subsp. *dysgalactiae* (SDSD) and *S. dysgalactiae* subsp. *equisimilis* (SDSE) which contain animal and human strains, respectively. Vieira and colleagues also supported their separation into these two subspecies, however, this research group recommended that differentiation was based on the haemolysis type produced, describing alpha-, beta- or non-haemolysis for the first subspecies and beta-haemolysis for the second one (Vandamme *et al.* 1996; Vieira *et al.* 1998). Based on clinical and epidemiological grounds, it was suggested that SDSD and SDSE should be distinguished as different species. However, the recognition between these two species was prohibited by the International

Committee on Systematic Bacteriology since no taxonomic indication support it (Kloos *et al.* 2001).

Salata and co-workers reported that SDSE is a common commensal of humans (Salata *et al.* 1989). In contrast, invasive infections caused by SDSE have been developing worldwide (Rantala 2014). SDSE is considered as the main aetiological agent of more than 80% of invasive infections caused by beta-haemolytic streptococci of groups other than A and B (Broyles *et al.* 2009). In several countries, the occurrence of bacteremia caused by SDSE has doubled or tripled and become as similar as the incidence of *S. pyogenes* (Rantala *et al.* 2009).

SSTIs are frequently caused by SDSE. These infections include abscesses, pyoderma, cellulitis, erysipelas, necrotizing soft tissue infections, surgical wound infections, and pyomyositis (Brahmadathan and Koshi 1989; Nohlgård *et al.* 1992; Bruun *et al.* 2013). In patients with cellulitis and erysipelas, isolation of GCGS is more frequent than *S. pyogenes* (Bläckberg *et al.* 2015; Bruun *et al.* 2015). SDSE can cause severe SSTIs such as necrotizing fasciitis and necrotizing myositis (Bruun *et al.* 2013). SDSE infections can be transmitted between two individuals and the majority of cases occur sporadically in nature rather than related to common source outbreaks. A rapid spread of SDSE infection might be associated with ecological contamination or mostly as a result of person to person close contact (Baracco 2019). There is an increased risk for injectable-drug users to be infected with cellulitis and skin abscesses caused by SDSE, and this is the main source of bacteremia in those patients (Craven *et al.* 1986). Individuals with burn infections are also vulnerable to SSTIs with SDSE; for example 8% of cutaneous group G streptococci burn infections were documented in one series (Brahmadathan and Koshi 1989; Rider and McGregor 1994). Many reports found that patients infected with SDSE severe diseases have streptococcal toxic shock syndrome (STSS) (Islam *et al.* 2016; Baxter and Morgan 2017; Baracco 2019).

1.6 Treatments for Bacterial Skin Infections

The proper management of skin bacterial infections depends on a good understanding of the clinical symptoms and the pathogens causing the infection (Sukumaran and Senanayake 2016). The use of antibiotics has led to an improvement in the treatment of most bacterial skin infections (Veien 1998). However, Sukumaran and Senanayake (2016) stated that antibiotic therapy is only essential for a spreading cellulitis or systemic infection. For example, incision

and drainage of the lesions could be used as a treatment of many cases infected with boils and carbuncles. Sukumaran and Senanayake (2016) also reviewed that skin infections caused by more than one organism can be treated by distinguishing between the two causative bacterial species such as cellulitis that caused by either *S. aureus* (staphylococcal cellulitis) or beta-haemolytic streptococci (streptococcal cellulitis). In such infections, blood or wound cultures can assist in the identification of the causative organism. Nevertheless, it can be difficult to differentiate between the two organisms in the absence of bacterial growth on cultures, and in order to protect against infections of both bacterial species, broad spectrum antibiotics are frequently used such as, cephalexin, flucloxacillin, dicloxacillin or clindamycin (Sukumaran and Senanayake 2016).

In terms of the recommended topical treatments, mupirocin is prescribed in cases of mild impetigo and folliculitis. It is a topical antibiotic which was initially isolated from *Pseudomonas fluorescens* (Gao et al. 2014). While the management of other infections has to be achieved by either incision and drainage or via using oral and intravenous antibiotics. Monotherapy with the topical fusidic acid has been correlated with the raised incidence of fusidic acid resistance among *S. aureus* strains (Howden and Grayson 2006; Williamson *et al.* 2014). Oral antibiotics can be used for patients who have no symptoms of systemic toxicity. Hospital evaluation is necessary for patients infected with serious skin diseases who have systemic symptoms, and such individuals require observation and intravenous antibiotics to treat their infections (Sukumaran and Senanayake 2016).

1.7 Antibiotic Resistance

Multiple antibiotics have a broad action on both commensal and pathogenic bacteria by killing or preventing the growth of microorganisms important for sustaining health, which have resulted in an increased incidence for the drug-resistant microbes to cause infection (Okhiria *et al.* 2009). Multiple infectious diseases are becoming multidrug resistant and are considered major public health problems across the world (Soleimani *et al.* 2010). The occurrence of antibiotic resistant pathogens is increasing worldwide. It has been recognized that the prolonged administration of antibiotics can cause imbalance of commensal microbiota in the body (Blaser 2011). The expansion of resistant bacteria is related to the administration of antimicrobials as growth promoters in animal production, as well as wide use of antimicrobials in humans. Most antibiotics are administered to patients before any diagnostic evidence based on cultures (Roghmann and McGrail 2006). In some parts of the world, antibiotics are losing their

effectiveness as a result of overuse and misapplication of them in both human and veterinary medicine (D'Souza *et al.* 2002). Thus, there is an urgent need to develop safe and effective alternatives to antibiotics for treating bacterial infections. Particularly, since skin infections comprise a large number of patients commonly presenting an emergency departments with skin abscesses and complications (Singer and Talan 2014), new treatments that do not promote antibiotic resistance are required.

1.8 Alternative Treatment Options

The possibility of infection recurrence has increased up to 60% due to antibiotic resistance in key pathogens, and this has led to the necessity to find safe long-term alternative options for the treatment of resistant infectious diseases (Bauer and van Dissel 2009) . In addition to the therapeutic activity of these alternatives, they can play a role in the reducing antibiotic-based medicine in human health. Since antibiotics have been so successful, limited attention has been paid to the usage of traditional treatments such as honey, essential oils, antimicrobial dressings, and probiotic microbes as safe natural medication for skin wounds (D'Souza *et al.* 2002; Okhiria *et al.* 2009). Due to the development of antimicrobial-resistant microbes, the scientific and clinical community have begun to use of probiotics for the prophylaxis and treatment of infectious diseases (Grounta *et al.* 2016). In addition, probiotic microorganisms can play a significant role alongside antimicrobial treatment to maintain a balanced the gut microbiota (Ohland and MacNaughton 2010).

1.9 Probiotic Bacteria

In 2001, the World Health Organization (WHO)/Food and Agricultural Organization (FAO) published the definition of probiotics in which they declared that probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the

host beyond basic nutrition" and this is the most commonly accepted definition of probiotics (Gomes da Cruz *et al.* 2009; De LeBlanc and LeBlanc 2014; Van Wyk *et al.* 2014). Iannitti and Palmieri (2010) pointed out that the ‘Probiotic’ term is a combination of the Latin and Greek words “*pro*” and “*bios*”, which mean together “*for life*”. Venugopalan and colleagues listed multiple descriptions that have been included in the modern definition of probiotics such as, probiotic drugs, probiotic food products (foods, food ingredients and dietary supplements),

designer probiotics (including genetically modified probiotics) and direct-fed microbials (probiotics for animal use) (Venugopalan *et al.* 2010).

A large number of clinical trials have provided evidence that specific strains of microorganisms possess health promoting properties. The frequent bacterial genera used in probiotic preparations has been reviewed by Heller (2001), and lactic acid bacteria (LAB) were described as the most common probiotic microorganisms used in commercial fermented and non-fermented dairy products in the present day. LAB include the genera *Lactobacillus, Bifidobacterium, Streptococcus, Lactococcus lactis,* some *Enterococcus* species (Ouwehand *et al.* 2002; Begovic *et al.* 2010). LAB can be present in different dairy products like yogurt, cheese and fermented milk, and in other fermented food products made from vegetables, fruits and meat. They are also existing in the urogenital system and gastrointestinal tract of humans and animals (Korhonen *et al.* 2009). The most widely studied probiotic strains include *Lactobacillus rhamnosus* GG (Jones 2010) and *Saccharomyces boulardii,* which is the only probiotic yeast used at the present time (Morrow *et al.* 2012). The use of probiotics has been increasing as they are generally regarded as safe (GRAS) with very few adverse side effects, as well as multiple studies now showing the advantages of these probiotics in both prevention and treatment of several infectious diseases (Kumar *et al.* 2015).

1.9.1 History of Probiotics

Clinical and health advantages of probiotics have been investigated many years ago. Eli Metchnikoff has started his research since 1908 studying the immunological effects of probiotics on human health and longevity (Jones 2010). Metchnikoff recommended that the consumption of specific bacteria present in dairy products and sour milk could assist an individual health by replacing the harmful bacteria in the body (Isolauri *et al.* 2002; Jones 2010). Recently, there is an increase in the number of existing products. Furthermore, the consumer knowledge with probiotic conception has developed. Thus, investigation into these products has also been expanded. Gomes da Cruz and associates found that during 2006, the ‘probiotic’ term was used in more than 600 products that were commercially available (Gomes da Cruz *et al.* 2009).

1.9.2 Delivery of Probiotics

There are various ways to deliver probiotics to consumers due to a diversity in industrial production methods, including dairy foods like fermented milks and cheeses, and non-dairy foods such as cereals, meat and olives, as well as tablets which comprise pure bacterial cultures.

Probiotics are available in markets as foods or dietary supplements (Venugopalan *et al.* 2010). They are also usually existed in yogurts, milks, pills and powders (Jones 2010). Yogurts are a major dairy product which typically contain *Lactobacillus* and *Bifidobacterium* as probiotic microorganisms. In the last few decades extra and putative probiotics cultures have been included in the contents of more than 80% of the yogurts that are sold in the United States (Sanders 2003). Companies interested in introducing new and healthy products have found that incorporation of probiotics into foods and beverages is an attractive marketing choice. Therefore, probiotic bacteria were added to an increased range of dairy and non-dairy foods/ beverages including, cheeses, frozen yogurts, cereal bars, juices and chocolates (Champagne *et al.* 2005).

Since probiotics are considered as effective ingredients in the food, there are few investigations which explore the impact of food structure on probiotic functions. Probiotic functions can be affected by the delivery vehicle in several ways, including cell structure variations and probiotic physiological condition, production of bioactive compound, fermentation end-products like organic acids and secondary metabolites such as bacteriocins. Incorporation of probiotic products in the diet and consumption incidence of products are also altered by the palatability of the delivery matrix (Sanders and Marco 2010). These factors also have a direct impact on probiotic cell fitness, product shelf life and stability. The quantity of active probiotic available in the product and its delivery to the consumer are affected by all of the above factors, and is an important consideration for manufacturers (Sanders 2008).

1.9.3 Health Benefits of Probiotics

Consumption of probiotics can provide the body with several health benefits which include, maintenance of the intestinal microbiota stability, relief of lactose intolerance, conservation of mucosal integrity (Salminen *et al.* 1998; Tang *et al.* 2010), boosting of the immune response (Cross 2002; De Bellis *et al.* 2010). Other advantages of probiotics intake involve, protection against infections, decreasing the risk of inflammatory bowel movements, prevention of diarrhoea from different causes, avoidance of chronic inflammation (Salminen *et al.* 1998), prevention of cancer (Wollowski *et al.* 2001; Commane *et al.* 2005), synthesis of vitamins, and protection from toxins (Ohland and MacNaughton 2010). Furthermore, different studies demonstrated that probiotics have advantages with gastrointestinal diseases like irritable bowel disease (IBD) and irritable bowel syndrome (IBS), urogenital tract infections (UTI), *Helicobacter pylori* infections (Teotia *et al.* 2014), skin and allergies (Iannitti and Palmieri 2010; Tomaro-Duchesneau *et al.* 2014). One of the most valued roles that probiotics have is the

prevention and treatment of several gastrointestinal diseases including IBS, IBD, antibiotic-associated diarrhoea (AAD) and dysbiosis (Shanahan and Quigley 2014; Wright *et al.* 2015). Due to the expanding knowledge about the benefits that probiotic bacteria provide to general health, the search for novel bacterial strains with probiotic action has become extensive. Both *in vitro* and *in vivo* procedures are necessary in the total preclinical investigation performed to confirm the health-enhancing features of probiotic bacterial candidates (Sorokulova 2008; Donovan *et al.* 2012; Papadimitriou *et al.* 2015).

1.9.4 Characteristics of a Probiotic Organism

Candidates need to be distinguished as probiotic bacteria are widely known to have many properties including, the ability to resist bile acid, gastric acidity and digestive enzymes. Furthermore, the capability to repress the growth of pathogenic microorganisms. The main characteristic of probiotics is that they have to be proven as safe and generally non-pathogenic (Vandenplas *et al.* 2015). They also have the ability to adhere to cells, reduce pathogenic bacteria adherents, coaggregate, and produce antimicrobial compounds such as hydrogen peroxide, acetoin, bacteriocins, and organic acids (lactic, formic, benzoic acids) (Delgado *et al.* 2001; Soleimani *et al.* 2010). To date, several studies have defined bacteriocins as proteinaceous antibacterial compounds that display bactericidal action against species closely related to the producer strain. Lactic acid bacteria, especially food-associated, produce important types of bacteriocins such as nisin, diplococcin, acidophilin, and bulgaricin that have been previously recognized and categorized in specific LAB species (Signoretto *et al.* 2000). Extracellular or membrane-associated active compounds are produced by many microorganisms and have essential roles in the maintenance of producing microorganisms. Biosurfactants are categorized mainly by their chemical structure and microbial origin. Rattanachaikunsopon and Phumkhachorn (2010) reviewed that the major classes of biosurfactants include glycolipids, lipopeptides, lipoprotein, phospholipids, fatty acids, polymeric biosurfactants and particulate biosurfactants (Kermanshahi and Peymanfar 2012). It has been stated by Ruiz -Moyano and colleagues that dairy fermented products are the most widely used source to provide probiotics, such as yogurt and fermented milk. Nonetheless, in recent times there is a necessity for novel and non-dairy probiotics. Therefore, conventional fermented foods may broaden the valuable progress of probiotic-type functional foods (Ruiz-Moyano *et al.* 2011).

1.10 The Use of Animal Models in Biological Research

Animals were introduced into research over a thousand years ago. Evidence indicates that even Aristotle, an ancient Greece philosopher and scientist had used animals in his studies to develop the understanding of living animals (Ericsson *et al.* 2013). The progress of animal models expanded during the 18th and 19th centuries when scientists carried out different animal experiments to explore the origin of life (Oparin 1957). Moreover, to improve the understanding of the human and animal anatomical, physiological, pathological and pharmacological features, animals were also used for more investigations in these fields. Having the opportunity to perform the experiments under controlled conditions and to imitate the biological circumstances of human and animal diseases, both supported the progress of scientific assays and the establishment of systematic animal models. Due to the emergence of animal models, most of the important information in a number of biological fields has been developed (Institute_of_Medicine and National_Research_Council 1988; Olson *et al.* 1991; Lieschke and Currie 2007). Model hosts' species which are mostly used in biomedical studies are the amoeba (*Dictyostellium discoideum* and *Acanthamoeba castellanii*), fishes (*Danio rerio* - zebrafish), frogs (*Xenopus*), the soil- living nematode (*Caenorhabditis elegans*), insects (*Drosophila melanogaster* - the fruit fly; *Bombyx mori* - the silk worm; and *Galleria mellonella* larvae - the greater wax moth) (Grounta *et al.* 2016). Multiple mammals including dogs, cats, monkeys, pigs, mice and rats are also widely used because of their closer relationship to humans (Olson *et al.* 1991).

Animals have a significant influence on quality of life and scientific research. For instance, Claude Bernard used animals to explain the pancreas function in digestion. They have also used by Albert Sabin to develop the oral live Polio virus vaccine. In addition, animals have recently contributed to understanding the pathogenicity of emerging virus infections such as Zika virus. Furthermore, animals play an important role in the surgical methods and anaesthesia procedures, as well as the advancement of new vaccines and drugs (Andersen and Winter 2019). Greek and Menache (2013) discussed that even with concerns about clinical relevance of animal data, the progress resulting from using animal models is extensive and trials using animals have been involved in approximately 90% of Nobel Prize research in medicine and physiology. To answer particular scientific questions and depending on the aim of different research, it is necessary to use the suitable animal model. In addition to that, the animal species should meet particular standards to be used as a model in the experiment. For example, the animal health

quality is essential for the achievement of reliable experimental results. Thus, researchers have to concentrate on using well treated and healthy animals (Andersen and Winter 2019). In parallel to the scientific importance, using animal in research studies has also provoked an argument and discussion on ethical considerations. The British parliament was one of the first to implement laws on the use of animals in research. The introduction of the Cruelty to Animals Act was introduced by the parliament in 1876. It contained regulations on animal-based investigations and emphasised three main points: 1. animal trials must only be conducted when these experiments are completely required for obtaining valuable information to reduce a pain, save or prolong life; 2. anaesthesia has to be done for experimental animals; and 3. if there would be an animal injury or suffering as a result of the procedure, the animal should be killed after the experimental method (Andersen and Winter 2019). The emergence of the 3R's Principle, to Reduce, Refine and Replace animal usage, was also started in the UK. William Russell, a zoologist and Rex Burch, a microbiologist produced a report which was subsequently published as a book explaining the 3R's principle (Russell *et al.* 1959). Each R indicates a principle for the animal ethics in experiments: **Reduction** is the performance of procedures that decrease the number of animals used per study. **Refinement** refers to the application of techniques which minimise the pain, distress or suffering of the research animals and that lead to their welfare improvement. For example, the application of non-aggressive procedures; the use of anaesthesia throughout the technique and analgesic routines to alleviate the pain during recovery; housing circumstances which deliver a relaxed and safe environment for the animal. **Replacement** is the main target for move away from animal use in science. It involves changing of animals with other models, like cell culture, invertebrate models, and using organ or cellular based systems. The 3R's Principles are now widespread and considered as a guidance for animal investigations in a lot of countries (Institute_of_Medicine and National_Research_Council 1988).

1.11 The Greater Wax Moth (*Galleria mellonella*) Larvae

The use of non-mammalian vertebrates and invertebrate animal species as *in vivo* models is economically and legally attractive (Dorer and Isberg 2006; Papadimitriou *et al.* 2015); furthermore, ethical issues are more limited with insect models. In terms of the evolutionary distance insects and vertebrates diversified over 500 million years ago, but there are still multiple similarities in their physiological and immunological characteristics (Boman and Hultmark 1987). Insects and mammals are similar in the structure and function of the innate immune system (Ratcliffe 1985; Klein 1997; Salzet 2001). There are several common features between

the innate immune response of insects and mammals (Hoffmann 1995; Fallon and Sun 2001) and the response of vertebrates to a microbial infection can be modelled by analysing the insect response to pathogens (Hoffmann 1995; Kimbrell and Beutler 2001). The primary line of protection in vertebrates is the innate immune response, therefore considerable attention has been undertaken on studying both the mammalian and insect response to infection, with robust associations between both systems well established (Salzet 2001).

The greater wax moth *Galleria mellonella* larvae, is a common insect model used in research. From this point forward, describing *G. mellonella* models will refer to the larvae. The term wax moth is a common name specified for different species of moths that have the ability to attack and destroy honeybee colonies and hive products (Williams 1997; Ellis *et al.* 2013; Kwadha *et al.* 2017). These are also called the wax (or bee) miller or the bee moth, and it can be also named as web (or wax) worm (Kwadha *et al.* 2017). One of these moths is *G. mellonella* which is a member of the subfamily Galleriinae within the family Pyralidae and the order Lepidoptera (Kavanagh and Reeves 2004; Kwadha *et al.* 2017). It is a beehive pest feeding upon pollen and damaging the weak or diseased hives in the combs (Kavanagh and Reeves 2004). *Galleria mellonella* life includes four developmental stages: egg, caterpillar, pre-pupa/pupa and adult insect (Figure 1.4).

Figure 1.4 Different developmental stages of *Galleria mellonella*. Eggs (1), approximately 10-day-old caterpillar (2), approximately 20-day-old caterpillar (3), 25-35-day-old caterpillar (4 and 5), approximately 40-day-old caterpillar (last larval stage) (6), pre-pupae and pupae (7 and 8), adult moths (9). Image adapted from (Jorjão et al. 2018).

Over the last two decades, this larva has been used as a model host to investigate for new drugs, explore host-pathogens interactions, and explain pathogenic infections comprising those caused by numerous gastrointestinal bacteria (Ramarao *et al.* 2012; Tsai *et al.* 2016). The effectiveness of antibiotic and antifungal treatments has also been assessed by using this model (Mesa-Arango *et al.* 2013; Benthall *et al.* 2015). Several animal studies have also been

successfully established in *G. mellonella* larva model including a study conducted to examine the prophylactic and therapeutic effect of *Lactobacillus* against candidiasis (Vilela *et al.* 2015) and another investigation performed to evaluate the therapeutic activity of LAB against *Listeria monocytogenes* infection (Upadhyay *et al.* 2016). Several studies of infectious diseases observed that the host response in *G. mellonella* and mammalian hosts are frequently positively associated with the virulence of pathogens such as *Listeria monocytogenes* (Mukherjee *et al.* 2010; Mukherjee *et al.* 2011), *Pseudomonas aeruginosa* (Jander *et al.* 2000; Miyata *et al.* 2003), *Staphylococcus aureus* (Desbois and Coote 2011), *Acinetobacter baumanii* (Peleg *et al.* 2009), *Yersinia pseudotuberculosis* (Champion *et al.* 2009), *Enterococcus faecalis* (Michaux *et al.* 2011), *Cryptococcus neoformans* (Mylonakis *et al.* 2005) and *C. albicans* (Cotter *et al.* 2000; Fuchs *et al.* 2010).

In contrast, there is limited information related to the use of microbes with health stimulating characters in the wax moth model. Two recent publications are available in this field, the first investigation was undertaken by Vilela and co-workers who explored the prophylactic and therapeutic properties of the probiotic strain *Lactobacillus acidophilus* ATCC 4356 in the larvae infected with *C. albicans*. They observed that the survival of the infected larvae increased after the injection with cells and supernatants of the probiotic strain. Decreased number of yeast cells in the larval haemolymph was also noticed which led to the attenuation of candidiasis in the model (Vilela *et al.* 2015). The second study detected that the treatment with cell-free supernatants of LAB and eugenol (either alone or in combination) improved the survival ratios of *G. mellonella* infected with *L. monocytogenes* and reduced the pathogen virulence. There was a significant decrease in several virulence factor traits such as pathogen invasion and adhesion to the intestinal cells, haemolysin production, and decreases in the expression of virulence genes (Upadhyay *et al.* 2016).

In relation to other invertebrate models, *G. mellonella* larvae *in vivo* models have a number of functional advantages, including their size, ranging between 1.5 - 2.5 cm that allows the simple handling and the delivery of accurate amounts of microbial cells by direct injection into the haemocoel through the last proleg. Furthermore, their low cost, easy maintenance, control and management, the possibility of performing large number of biological and technical replicates per test and quick experimental inoculation and monitoring make them a favourable model. Finally, their ability to survive at 37°C which is important for the investigation of multiple human pathogens that persist at human body temperature (Cotter *et al.* 2000; Scalfaro *et al.* 2017).

1.12 Thesis Aims

The overall aims of this PhD were to examine the *in vitro* antibacterial effect of lactic acid bacteria (LAB) isolated from fermented dairy and non-dairy products against the major causatives of skin diseases, and to explore the therapeutic potency of these food-based bacteria against skin infections using the Greater wax moth *Galleria mellonella* larvae as an *in vivo* model. The main goals were achieved by the following objectives:

1. To investigate the value of fermented food products as isolation sources of LAB species with antibacterial effects, and to explore the genomic contents of these isolates and skin pathogens involved in this research (Chapter 3).
2. To study the bacterial diversity (including LAB) of probiotic food samples using metagenomic and metataxonomic approaches. Furthermore, to compare the findings of NGS technique with those of culture-dependent method (Chapter 4).
3. To investigate the *in vitro* antagonistic activity of LAB species against three main pathogenic species including, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Streptococcus dysgalactiae* subsp *equisimilis* (Chapter 5).
4. To examine the susceptibility of *G. mellonella* larvae to the injected bacterial species, and to evaluate the therapeutic effect of food-borne *Lactobacillus* species against the pathogenic bacteria using *G. mellonella* as an *in vivo* infection model (Chapter 6).

1.13 References

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Chapter Two

General Materials and Methods

2 General Materials and Methods

2.1 General Materials

All the general culture media and reagents used to perform the experiments in each chapter are illustrated in Table 2.1 with their make, supplier and purpose of use.

Table 2.1 Media and materials used in the study

Materials	Supplied Companies	Purpose of Use	References
DeMan Rogosa and Sharp Medium (MRS) (Broth and Agar)	Lab M Ltd (Neogen) / UK	Cultivation of *Lactobacillus* Species	(De Man *et al.* 1960)
Trypticase Soy Broth (TSB) and Agar (TSA) Media	Oxoid Ltd / UK	Cultivation of Pathogenic Species	(Lennette *et al.* 1985; Mac Faddin 1985)
Fastidious Anaerobe Broth Medium (FAB)	Lab M Ltd (Neogen) / UK	Inoculation of Food Samples for DNA Extraction	(Ganguli *et al.* 1982; Ganguli *et al.* 1984)
Muller-Hinton Agar Medium (MHA)	Oxoid Ltd / UK	Determination of the Antagonistic Activity	(Mueller and Hinton 1941)
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich Ltd / UK	Preparation of Stock Bacterial Cultures	
Guanidine Isothiocynate solution	Invitrogen / US	Bacterial DNA Extraction	
Zirconia/Silica Beads (0.1 mm) diameter	Thistle Scientific (BioSpec Products)	Bacterial DNA Extraction	
PCR Nucleotide Mix	Promega / UK	Bacterial DNA Extraction	
1kb DNA ladder	Promega / UK	Gel Electrophoresis	
Blue/Orange 6X Loading Dye	Promega / UK	Gel Electrophoresis	
Hyper Ladder 1kb	Bioline Reagents Ltd / UK	Gel Electrophoresis	
Grade Agarose Gel	Severn Biotechnology Ltd / UK	Gel Electrophoresis	
TAE 1X Buffer solution	Severn Biotechnology Ltd / UK	Gel Electrophoresis	
Phosphate Buffered Saline (PBS) 1X (0.01 M)	Severn Biotechnology Ltd / UK	Preparation of Bacterial Dilutions	
Cyclohexamide 100 mg/l	Melford Biolaboratories Ltd / UK	Isolation of *Lactobacillus*	
Phosphate Buffered Solution (1.0 M)	Sigma-Aldrich Ltd / US	Neutralization of *Lactobacillus* Cultures	
Sodium Hydroxide NaOH (1M)	Fisher Scientific Ltd / UK	Neutralization of Cell Free Supernatants	

2.2 Collection and Culturing of Bacterial Species

2.2.1 Collection and Culturing of Lactic Acid Bacterial Strains

Eight *Lactobacillus* strains were obtained from the Marchesi Laboratory culture collection at the Organisms and Environment Division, School of Biosciences, Cardiff University. Other strains were provided from different culture collections such as, German Collection of Microorganisms and Cell Cultures (DSMZ), Institute d'Oenologie de Bordeaux (IOEB), National Collection of Industrial, Food and Marine Bacteria (NCIMB), American Type Culture Collection (ATCC), Belgian Co-ordinated Collections of Microorganisms (BCCM/LMG), National Collection of Type Cultures (NCTC) and Cultech Ltd (Cul). All the strains are presented in Table 2.2. In addition to the above strains, several *Lactobacillus* species were isolated from fermented food products (Chapter 3).

De Man Rogosa and Sharp (MRS) broth and agar (Lab M) were used for growing *Lactobacillus* strains which were incubated aerobically or anaerobically at 37°C for 24-72 h in the anaerobic cabinet (Electrotek). *Bifidobacterium* strains were cultured on MRS agar supplemented with 0.05% (w/v) L-cysteine hydrochloride (Sigma-Aldrich) and incubated anaerobically at the same temperature. *Lactococcus* and *Streptococcus* species were cultivated using M17 broth (Sigma-Aldrich) and agar (Fisher Scientific) supplemented with 10% (w/v) sterilised lactose solution. Both species showed good growth after incubation aerobically for 48 h.

Table 2.2 Reference bacterial strains used in this study

Bacterial Species Name	Strain Number	Strain Symbol	Isolation Source
Lb. fermentum	DSM 20055	Lb1	Saliva
Lb. casei	Imunitass	Lb2	Probiotic Yakult Drink
Lb. casei	Shirota	Lb3	Probiotic Yakult Drink
Lb. brevis	IOEB 9809	Lb4	Bordeaux Wine
Lb. brevis	IOEB 8907	Lb5	Red Wine
Lb. plantarum	NCIMB 8826	Lb6	Human Saliva
Lb. plantarum	NCIMB 8826	Lb7	Human Saliva
Lb. reuteri	ATCC 23272	Lb8	Adult Intestine
Lb. reuteri	ATCC 23272	Lb9	Adult Intestine
Lb. hilgardi	IOEB 9648	Lb10	Sweet White Wine
Lb.acidophilus	BCCM/LMG 19170 (Type)	Lb26	Cider
Lb.casei	BCCM/LMG 6904 (Type)	Lb27	Cheese
Lb. brevis	BCCM/LMG 6906 (Type)	Lb28	Human Faeces
Lb. plantarum	BCCM/LMG 6907 (Type)	Lb29	Pickled Cabbage
Lb. paracasei subsp *paracasei*	BCCM/LMG 7955	Lb30	Not Applicable

Lb. gasseri	DSM 20077	Lb32	Human Faeces
Lb. crispatus	DSM 20584 (Type)	Lb33	Eye

DSM: German Collection of Microorganisms and Cell Cultures; IOEB: Institute d'Oenologie de Bordeaux; NCIMB: National Collection of Industrial, Food and Marine Bacteria; ATCC: American Type Culture Collection; BCCM/LMG: Belgian Co-ordinated Collections of Microorganisms; NCTC: National Collection of Type Cultures and CUL: Cultech Ltd

2.2.2 Collection and Culturing of Pathogenic Bacteria

Three pathogenic clinical isolates were collected from the Department of Dermatology, University Hospital of Wales, Cardiff, Public Health Wales (PHW). They were isolated from different body sites of patients who were infected with skin diseases. All pathogenic isolates were previously identified by cultural characteristics on selective media as one isolate of *Staphylococcus aureus* (SA1) and two isolates of the genus *Streptococcu*s, the first isolate belonged to the serological Group A Streptococci (GAS) and the second one was from the Group G Streptococci (GGS). These were isolated from perineal area, non-specified wound and leg, respectively. Table 2.3 showed the antibiotics sensitivity of these isolates recorded by the hospital. Trypticase soy broth (TSB) and agar (TSA) (Oxoid) were used as the growth culture media for pathogenic bacteria.

Table 2.3 Antibiotics sensitivities of clinical pathogenic isolates

Antibiotics Tested	**Clinical Pathogenic Isolates**		
	***Staphylococcus aureus* (Perineal area)**	***Streptococcus* A (Non-specified wound)**	***Streptococcus* G (Leg)**
Amoxicillin	Not tested	S	S
Cefoxitin	S	Not tested	Not tested
Clarithromycin	S	S	S
Clindamycin	S	S	S
Doxycycline	S	S	S
Erythromycin	S	S	S
Flucloxacillin	S	Not tested	Not tested
Fusidic acid	S	Not tested	Not tested
Gentamicin	R	Not tested	Not tested
Levofloxacin	Not tested	S	S
Penicillin	Not tested	S	S
Tetracycline	S	S	S
Trimethoprim/Sulfonamides	Not tested	S	S

2.3 Storage of Bacterial Cultures

Freshly grown bacterial colonies were removed from plates and inoculated in broth cultures. Stocks of lactic acid bacteria and pathogens were prepared by adding 8% (v/v) of dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to an overnight broth culture, with duplicate stocks made for each individual isolate. All the stock cultures were maintained at -80°C in cryogenic vials (Thermo Scientific).

2.4 Identification of Bacterial Species

2.4.1 Bacterial DNA Extraction

Type strains of *Lactobacillus*, food isolates of *Lactobacillus* and all the pathogenic isolates were streaked from stocks on agar media, grown up to colonies and inoculated into liquid media (5 mL in a 15 mL tube) prior to growth at 37°C for 24 h with gentle rocking. The cultures were centrifuged at 1000 g for 10 min and the supernatant was discarded. To induce lysis, the pellet was re-suspended in 800 µl of 4 M guanidine isothiocynate solution (Invitrogen), and mixed by vortex for 2 min (Pitcher *et al.* 1989). Two millilitres microtubes tubes (Star Lab) were prepared for each extraction by adding 1 g of zirconium/silica beads (0.1 mm) (Thistle Scientific) into each tube. After that, 800 µl of the bacterial suspension were transferred into the tubes and they were loaded onto beads beater instrument (homogenizer – Fastprep 24, MP Biomedicals, USA). The bead beating was carried out three times at setting 6.5 for 30 sec, and the tubes were placed in ice between subsequent beating steps for 5 min. The tubes were centrifuged at 18800 g for 2 min and the supernatant was used for DNA extraction with a Maxwell 16 Tissue Purification Kit (Promega), using the automated Maxwell DNA extraction instrument (Promega), following the manufacturer's instructions. The extracted DNA (approximately 300 µl) was collected in 1.5 ml non-stick microcentrifuge tubes; 30 µl of the solution was kept in an extra microcentrifuge at 4°C to enable PCR while the remaining was stored at -20°C.

2.4.2 Polymerase Chain Reaction (PCR) of the 16S rRNA gene

A partial sequencing of 16S rRNA gene was amplified using bacterial universal primers 27F (5`-AGAGTTTGATCCTGGCTCAG-3`) and 1492R (5`-GGTTACCTTGTTACGACTT-3`) (Eurofins Genomics) (Lane 1991). The amplification reaction of the 16S rRNA gene was

carried out in 0.2 ml PCR tubes. PCR Nucleotide Mix (Promega) was used for all PCRs. Each PCR reaction consisted of the following, 3 µl $MgCl_2$ (25 mM), 5 µl 10X reaction buffer (without 15 mM $MgCl_2$), 1 µl dNTPs (10 mM each), 5 µl (10 pmol/µl) of upstream (forward) and downstream (reverse) primers' solution, 0.25 µl *Taq* DNA polymerase (5 U/µl), 2 µl of template DNA (concentration unknown), and sterile water to a volume of 50 µl. The PCR amplification has initial DNA denaturation at 95°C for 50 sec, followed by one cycle of denaturation at 94°C for 30 sec, 35 cycles annealing at 55°C for 30 sec and extension at 72°C for 1.5 min, which was followed by a final extension at 72°C for 5 min. At the end of the reaction, the tubes of PCR products were kept at 4°C to perform gel electrophoresis.

2.4.3 Gel Electrophoresis

Prior to loading 5 µl of the 1 kb DNA ladder (Promega) was mixed with 2 µl of blue/orange 6X loading dye (Promega) and 5 µl of each PCR product was also mixed with same amount of the loading dye. Hyper ladder 1 kb (Bioline) was used as well. Electrophoresis was performed in a 1.2 % (w/v) agarose gel with TAE 1X buffer (Severn Biotechnology), and DNA separated at 100 volts for 40 min. Products were visualized by UV using a VersaDoc (Biorad).

2.4.4 Identification of Bacterial Species

To identify food *Lactobacillus* isolates and to confirm the identification of type *Lactobacillus* strains, as well as clinical pathogens, PCR products of the 16S rRNA gene amplification were sequenced by Macrogen Europe Laboratory (the Netherlands) and Eurofins Genomics (Germany). To establish the taxonomy at the species level, the obtained sequences were compared to the National Center for Biotechnology Information (NCBI) data base (NCBI_Resource_Coordinators 2018) using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1990) and Ribosomal Database Project (RDP) (Maidak *et al.* 2000).

2.5 Estimation of the Bacterial Viability

In cultures of LAB and pathogenic isolates, determination of bacterial cells' concentration was performed by standard colony forming units (CFUs) counting (Schellenberg *et al.* 2006). Bacterial species were revived on agar media and the plates were incubated at 37°C for 18 h. Three to five single colonies were selected from the solid cultures of each isolate and inoculated into 3 mL broth media in a 15 mL falcon tube (MRS broth was used for LAB species and TSB for pathogens). After incubation (37°C for 18 h), serial decimal dilutions of the cultures

were made using sterile phosphate buffered saline (PBS) 1X (0.01 M) (Severn Biotechnology) resulting in a final dilution of 10^{-8}. Ten microliters of each dilution were plated three times onto solid media. The plates were allowed to dry at room temperature and incubated at 37°C for 24-72 h. Numbers of colony forming units (CFUs) were counted in each dilution using the colony counter (Stuart Scientific) (Miles *et al.* 1938). To calculate the number of bacterial cells per ml of the original culture, the following equation was used:

No of CFU/ml = (Average number of the colonies from 3 drops × Inverse of the dilution factor) × (1000 µl ÷ volume dropped onto plate (e.g., 10µl))

To confirm the approximate numbers of CFUs/ml of the initial bacterial cultures for both LAB and pathogenic bacteria, this procedure was repeated before each individual experiment which was done to evaluate the *in vitro* antagonistic activity (Chapter 5) and the *in vivo* therapeutic effect of *Lactobacillus* species against the pathogens (Chapter 6).

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Chapter Three

Isolation and Characterisation of Bacterial Species

3 Isolation and Characterisation of Bacterial Species

3.1 Introduction

Bacterial species involved in the present research which were from two groups and included, clinical isolates of pathogenic bacteria which cause skin infections, and a collection of *Lactobacillus* type strains. In addition to these, isolation of lactic acid bacteria was conducted from fermented food products to determine the bacterial species within these samples, and to explore the antibacterial potential of the isolates against pathogenic bacteria. Characterisation of both LAB and pathogenic isolates was performed using 16S rRNA gene analysis to establish their taxonomic classification to the species level. Analysis of draft genomes was carried out for all isolates, to explore their genomic features. In LAB genomes, genes encoding for several compounds that may contribute in the antibacterial activity were investigated. Regarding the pathogenic bacteria, genomes were examined for the presence of genes associated with virulence factors which play a role in the pathogenicity.

Fermented foods and beverages contain numerous functional microbes including various species of LAB, which are recognised for probiotic and antimicrobial features (Rezac *et al.* 2018). The American Food and Drug Agency (FDA) approved LAB as generally regarded as safe (GRAS) bacteria. The Qualified Presumption of Safety (QPS) status has been granted by European Food Safety Authority (EFSA) for a large number of LAB genera including, *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc* and a few *Streptococcus* species (Goel et al. 2020). Due to the health-stimulating influences of *Lactobacillus* strains, increasing attention on commercial application of these strains isolated from classical naturally-fermented dairy products such as cheese, yogurt and fermented milk, has arisen. Nonetheless, it is necessary to develop non-dairy probiotic products because of the growing number of consumers having milk protein allergy and those with various nutritional habits (e.g., veganism) (Gupta and Abu-Ghannam 2012). As a promising alternative, table olives are plant-based fermented products that have been described for their several valuable biological advantages, as these products contain numerous health promoting microbial species (Lavermicocca *et al.* 2005; De Bellis *et al.* 2010). *Lactobacillus* strains in a food product must conquer chemical and physical barriers in the gastrointestinal tract to deliver their health benefits (Del Piano *et al.* 2006). These strains must have the ability to tolerate the intestine acidic environment and overcome bile stress. Furthermore, probiotic bacteria should be capable of surviving within food products in sufficient

numbers during production and storage stages of these products (Ljungh and Wadstrom 2006). Dietary probiotic supplementation mostly includes dairy products, though, probiotics can be also integrated into non-dairy fermented food products, exhibiting an alternative source of new probiotic strains (Kerry *et al.* 2018). The flavour of dairy products is influenced by the bacterial enzymes available in these products. Although most of these enzymes are undesirable, LAB produce enzymes with an essential importance (Mensah *et al.* 1991) that affect product features, particularly flavour and texture in yogurt and cheese (Giraffa 2014). Due to their significant antibacterial capability, LAB have been broadly used in foods. Among the antibacterial substances reported in previous investigations, short peptides (Muhialdin *et al.* 2018), organic acids (Reis *et al.* 2012) and fatty acids (Ogawa *et al.* 2005) play important roles in the antibacterial efficacy, in addition to other compounds, e.g. hydrogen peroxide.

The 16S ribosomal RNA (rRNA) gene has nine variable regions which are usually used to differentiate among bacterial species. Sequence in the 16S rRNA gene varies between each two regions and no individual variable region of this gene is appropriate to distinguish among all bacteria (Kumar *et al.* 2011). The entire size of 16S rRNA gene is approximately 1500 bp. However, recent amplicon sequencing chemistries and platforms sequence fragments between 200 to 500 bp (Kircher and Kelso 2010). Using this fragment, a target organism can be assigned to a taxonomic group. The genera of most bacteria are usually identified depending on the V1, V2, V3 and V6 regions which are recommended for identification to the genus level (Chakravorty *et al.* 2007; Guo *et al.* 2013).

Investigation of the genus *Lactobacillus* has revealed a remarkable potential for both health and food industry. Recent projects which rely on genome sequencing have helped to lead to an improved characterisation of the genus (Klaenhammer *et al.* 2002). Schleifer and Ludwig (1995) reported that the *Lactobacillus* genus is highly diverse and its phylogeny is relatively complicated. Depending on 16S rRNA gene phylogeny, *Lactobacillus* species were divided by these researchers into three groups: *Leuconostoc* group, *L. casei-Pediococcus* group and *Lactobacillus* group. It was also stated by Dellaglio and Felis (2005) that the phylogeny of *Lactobacillus* species is difficult to relate to their phenotypes, like metabolic features. Recognition of new species and classification methods resulted in frequent reclassifications and an imprecise taxonomy (Schleifer and Ludwig 1995; Dellaglio and Felis 2005). Application of genome sequencing supports the current phylogenetic structure, from numerous genes or proteins (Claesson *et al.* 2007). The lactic Acid Bacteria Genome Consortium (LABGC) collaborated with the US Department of Energy-funded Joint Genome Institute (JGI) to

introduce the most impressive LAB sequencing project in 2001. The genome sequence of eleven various LAB were published by the JGI group, such as *L. delbrueckii* ssp. *bulgaricus*, *L. brevis*, *L. casei* and *L. gasseri* (Makarova *et al.* 2006).

The capacity of pathogenic bacterial species to cause a wide range of infections is associated with several extracellular and cell wall virulence factors. These factors are expressed in a coordinate manner during different stages of infection and include, colonization, invasion or avoidance of host immune system, cell growth and reproduction, bacterial spread and production of harmful toxic effects in the host (Holmes *et al.* 2005; Torres *et al.* 2010; Bien *et al.* 2011). Adherence of bacteria to epithelial cells is the initial stage required for colonisation of a new host. The bacterial attachment process consists of two steps, a primary non-specific interaction with the epithelial tissues, followed by a high affinity interaction with proteins of the host extracellular matrix (ECM) (Kreikemeyer *et al.* 2004; Reglinski and Sriskandan 2015). The expression of numerous tissue-specific adhesins is required for adherence, to prevent the bacterial elimination by the flow mechanisms of mucous and other body fluids (Cunningham 2000; Courtney *et al.* 2002). Adhesins also assist in the progress of the pathogen-ECM interactions and facilitate the bacterial incorporation into host cells resulting in long-lasting persistence and contributing in the bacterial pathogenesis (Kreikemeyer *et al.* 2004). Adhesins are divided into two groups, cell-wall-anchored surface proteins which are covalently attached to peptidoglycans of the bacterial cell wall. These are termed the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Speziale *et al.* 2009). The second group of adhesins are noncovalently anchored to the cell surface, which are secreted proteins but re-bind to the bacterial cell surface. These are named as secretable expanded repertoire adhesive molecules (SERAMs) (Chavakis *et al.* 2005). Both types of protein are included in the bacterial colonization within host tissues and in the evasion of host immune response (Patti *et al.* 1994; Chavakis *et al.* 2005). Fibronectin (Fn) is one of the host ECM proteins, it is a high-molecular-weight glycoprotein which plays a significant role in several cellular processes including phagocytosis and substrate adhesion. As fibronectin has the ability to attach to various receptors and substrate molecules, this binding characteristic has been utilised by numerous bacterial species to facilitate colonization and invasion steps of the host tissues (Ozeri *et al.* 1996). Other important proteins of the host ECM or plasma including, fibrinogen, laminin and collagen (Kreis and Vale 1999; Cheung *et al.* 2002).

3.2 Aims

1- Using food fermented products including yogurt and olives as isolation sources for lactic acid bacteria and exploration of the LAB species available in these products.

2- Classification of food-based lactobacilli and pathogenic isolates using the 16S rRNA gene sequence, and identification of the bacterial species.

3- Application of the whole genome sequencing strategy to obtain draft genomes of both food-isolated lactobacilli and pathogenic species.

4- Performance of the genomic analysis of food-based lactobacilli to investigate the genes encoding for antibacterial compounds responsible for the inhibitory activity of these isolates against pathogens.

5- Performance of the genomic analysis of skin pathogenic species to detect the genes encoding for virulent factors associated with the pathogenicity.

3.3 Methods

3.3.1 Isolation and Identification of Bacterial Species

3.3.1.1 Isolation and Identification of Lactic Acid Bacteria from Food Products

Lactic acid bacteria were isolated from three fermented food products. The first product was thick yogurt (Labneh/Pinar), while both of the second and third products were olives (Zer and Altunsa). All the products were purchased from the local supermarkets in the city of Baghdad/Iraq. Samples were aseptically transferred to the laboratory in sterile containers. 15 ml Falcon tubes containing MRS broth medium were inoculated by each sample in a percentage of 1% v/v and incubated anaerobically (Section 2.2.1) at 30°C for 48 h. For isolation of lactic acid bacteria, Pour-plate method was performed. A series of 10-fold dilutions were prepared for each cultured sample using sterile phosphate buffered saline (PBS) 1X (0.01 M). One ml was transferred from each of the last three dilutions into sterile empty petri dishes. Melted sterile MRS agar contained 100 mg/l cyclohexamide (to prevent the growth of saprophytic fungi) was poured on the transferred volume and mixed by rotating the plates which were left to solidify at room temperature for a couple of minutes and incubated anaerobically at 30°C for 72 h. Based on colony morphology (shape and colours), representative single colonies with typical *Lactobacillus* characteristics (circular, oval, spindle shaped, smooth edge, creamy coloured) were randomly selected from each plate and assessed by Gram stain and cell morphology. These colonies were sub cultured several times on the same medium by streaking to obtain pure colonies. After that, the growing bacteria were inoculated in MRS broth and incubated aerobically under the same growth conditions to prepare stock cultures of the isolated colonies (Doulgeraki *et al.* 2013; Guetouache and Guessas 2015). The obtained isolates were identified by sequencing their 16S rRNA gene (see chapter 2, sections 2.4.1-2.4.4).

3.3.1.2 Collection and Identification of Clinical Pathogenic Species

Collection of pathogenic isolates was carried out as described in chapter 2 (see section 2.2.2) and identification of these isolates was performed by 16S rRNA gene sequencing (see chapter 2, sections 2.4.1-2.4.4).

3.3.2 Whole Genome Sequencing (WGS) of the Bacterial Isolates

Genomic DNA was extracted from food isolated lactobacilli and pathogens using Maxwell 16 Tissue Purification Kit (Promega) and Maxwell instrument (see chapter 2, section 2.4.1). DNA samples were given to the Genomics Hub. A Neoprep protocol from Illumina was used according to the manufacturer's instructions. Sequencing was carried out on Nextseq 500 (2 x 150bp) paired end sequencing and approximately 180 Million reads produced for analysis. Raw sequence data for each isolate was run through a software pipeline to obtain draft genomes. These programs including, FastQC, TrimGalore, Flash, SPAdes, BWA, Pilon, QUAST and Prokka. The pipeline was run using a BASH script in CLIMB linux virtual servers (Appendix 1).

3.4 Results

3.4.1 Isolation and Identification of Bacterial Species

3.4.1.1 Isolation of Lactic Acid Bacteria from Fermented Food Products

Three fermented food products were used as sources for lactic acid bacteria isolation. The first product (P1) was a thick yogurt (labneh) manufactured by Pinar (Figure 3.1), the second and third products (P2 and P3) were olives manufactured by Zer and Altunsa (Figures 3.2 and 3.3). After performing pour-plate method and incubation period, a total of ten isolates were obtained from those samples. Isolates were Gram-positive, not spore-forming and short rod or coccobacilli shaped. These isolates were selected for identification and antagonism analysis. Figure 3.4 presents different shapes (round, oval, and spindle) of growing colonies of lactic acid bacteria under the surface of MRS agar medium, which are isolated from the labneh yogurt by pour-plate method. Whereas Figure 3.5 shows the cultural morphology of the single colonies, these are round, soft margin and creamy colour.

Figure 3.1 The first food product (P1) for LAB isolation, thick yogurt (Labneh/Pinar).

Figure 3.2 The second food product (P2) for LAB isolation, olive (Zer).

Figure 3.3 The third food product (P3) for LAB isolation, olive (Altunsa).

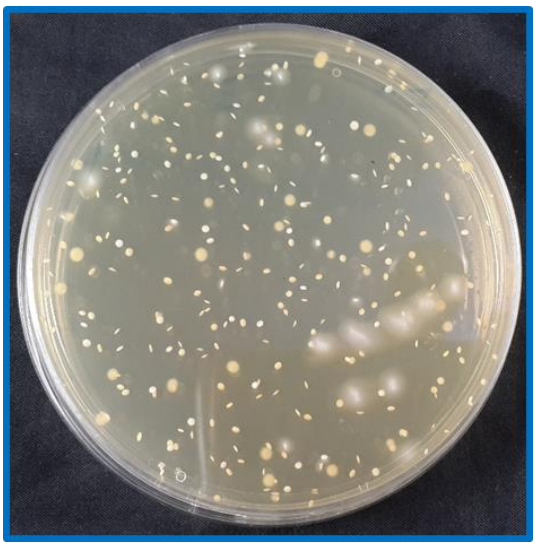

Figure 3.4 Cultural morphology of lactic acid bacteria growing under the surface of MRS agar by pour-plate method.

Figure 3.5 Cultural morphology of lactic acid bacteria growing on the surface of MRS agar by spread-plate method.

3.4.1.2 Identification of Lactic Acid Bacterial Species

Genomic DNAs were extracted from *Lactobacillus* type strains and food lactobacilli isolates using a Promega Maxwell automated DNA extraction platform and the 16 Tissue Purification Kit. After the performance of PCR for the extracted DNAs using 27F and 1492R primers, PCR products were visualized by agarose gel electrophoresis. All these isolates showed clear bands with an approximate size of 1500 bp (Figures 3.6 and 3.7).

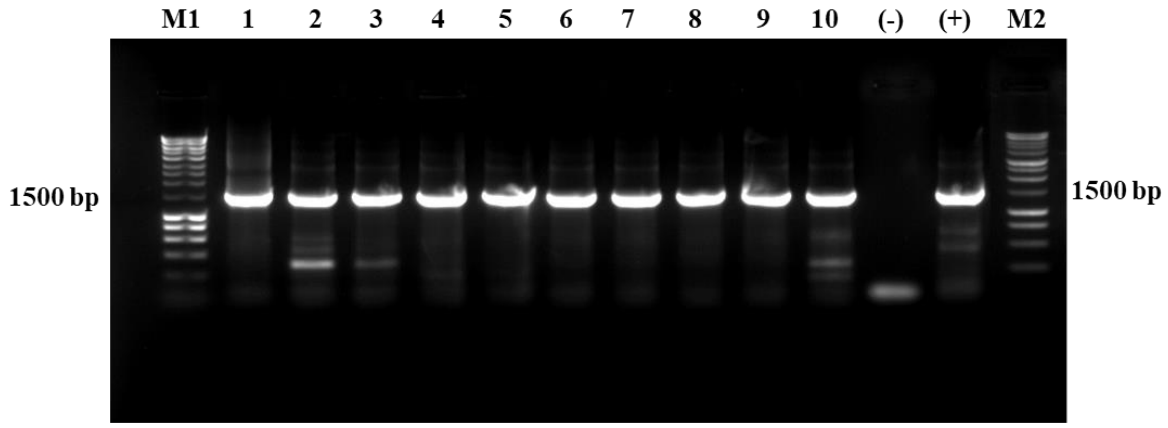

Figure 3.6 Agarose gel electrophoresis of PCR products of type *Lactobacillus* type strains; Lane M1: Marker 1 (hyper ladder1kb), Lanes 1-10: type *Lactobacillus* strains, Lane M2: Marker 2 (ladder 1kb), (-): -ve control, (+): +ve control.

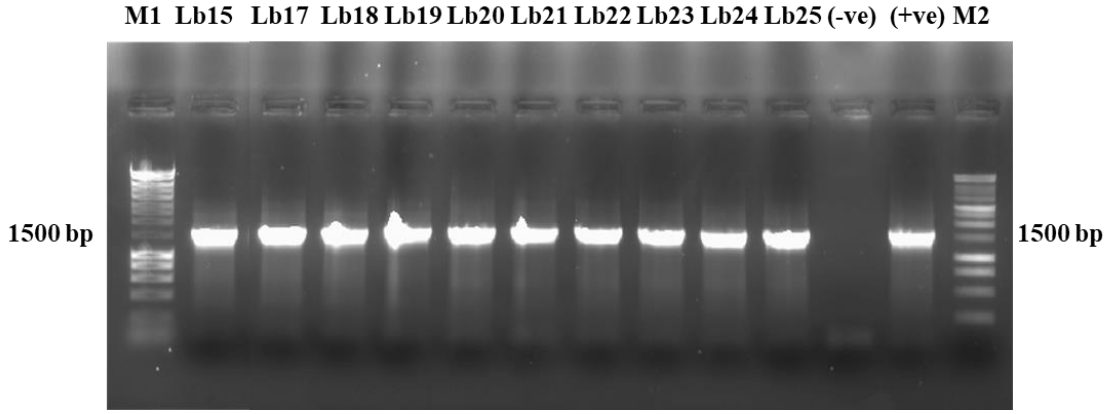

Figure 3.7 Agarose gel electrophoresis of PCR products of food *Lactobacillus* isolates; Lane M1: Marker1 (hyper ladder1kb), Lanes 15-25: food *Lactobacillus* isolates, Lane M2: Marker 2 (ladder 1kb), (-): -ve control, (+): +ve control.

After the performance of 16S rRNA gene sequencing of *Lactobacillus* type strains, nucleotide sequences were compared with the existed databases by using both BLAST at the NCBI and the Sequence Match tool at the RDP, to assess their taxonomic classification and compare the results to genus and species names stated in the bacterial stock manifest. Table 3.1 depicts the species names of *Lactobacillus* type strains in the stock culture and their identification using the two database tools. Both Lb1 and Lb6 strains showed the highest sequence similarities. In BLAST, these two strains were identified as the same reported species in bacterial stocks with identity percentages of 93% and 92%, respectively. These isolates revealed similar results using the RDP tool with sequence matching scores of 0.407 for Lb1 and 0.283 for Lb6. Regarding the strains Lb2, Lb9 and Lb10, these isolates were classified under the

genus *Lactobacillus* in both databases. The Lb2 strain was previously reported as *Lb. casei* in stocks, and was identified under the same species in RDP. Nevertheless, it was recognised as *Lb. rhamnosus* GG in BLAST. Ward and Timmins (1999) mentioned that *Lb. casei* and *Lb. rhamnosus* GG are closely related taxonomic species within heterofermentative lactobacilli, and it is difficult to differentiate between them by the conventional fermentation profiles. Classification of Lb9 strain in both tools presented different species to that documented in stocks. Although Lb10 strain was recognised as *Lb. hilgardii* in stocks, it was identified as *Lb. brevis* by the two tools. The strain Lb4 was documented as *Lb. brevis* IOEB 9809 in stocks, nevertheless, it was identified as fungal species which were *Carnobacterium divergens* and *Beauveria bassiana* using BLAST and RDP, respectively. Identification of fungal species depends on the performance of 18S rRNA gene sequencing, however, due to the sequence similarity of both 16S rRNA and 18S rRNA genes, bacterial universal primers might show a product of a fungus when fungal DNA is abundant which resulted in occurrence of non-specific amplification. Based on the data provided, *Lactobacillus* type strains exhibited relatively low sequence similarities to lactic acid bacterial species depending on 16S rRNA gene sequence. These findings seem to be consistent with previous research, which found that a group of isolates had similar phenotypic features to those of lactobacilli members. However, they revealed low 16S rRNA gene sequence resemblance to the known species of LAB and some isolates represented novel species (Endo and Okada 2007). In contrast, Kermanshahi and Peymanfar (2012) described nucleotide base sequences of *Lactobacillus* spp. 16S rRNA gene as a precise source for phylogenetic identification and analysis.

The classification of the ten food-based *Lactobacillus* isolates was undertaken using the same two databases. The obtained sequences from both BLAST and RDP tools revealed similar species for the individual isolate. The four bacterial species isolated from yogurt were identified as *Lb. delbrueckii*, while the other six species isolated from olive samples were recognised as *Lb. plantarum*. It was also observed that the reverse DNA strand had a higher quality of nucleotides alignments rather than the forward strand as indicated from the identity percentages and s-ab scores in BLAST and RDP, respectively (Table 3.2).

Table 3.1 Re-identification of type *Lactobacillus* strains using BLAST and RDP tools

Strain Symbol	Strain Name in the Stock	The Closest Hit in BLAST Report	Identity Percentage in BLAST	The Closest Hit in RDP Report	S-ab Score in RDP
Lb1	*Lb. fermentum* DSM20055	*Lb. fermentum*	93%	*Lb. fermentum*	0.407
Lb2	*Lb. casei* imunitass	*Lb. rhamnosus* GG	92%	*Lb. casei*	0.419
Lb3	*Lb. casei* Shirota	*Lb. casei*	87%	*Oedogoniomyces* sp (fungi)	0.244
Lb4	*Lb. brevis* IOEB 9809	*Carnobacterium divergens* (fungi)	85%	*Beauveria bassiana* (fungi)	0.229
Lb5	*Lb. brevis* IOEB 8907	*Lb. yonginensis*	95%	*Kazachstania naganishii* (fungi)	0.229
Lb6	*Lb. plantarum* WCFS1 (NCIMB8826)	*Lb. plantarum*	92%	*Lb. plantarum*	0.283
Lb7	*Lb. plantarum* WCFS1 (NCIMB8826)	No Hit	NA	*Coelomomyces stegomyiae* (fungi)	0.263
Lb8	*Lb. reuteri* F275/ ATCC23272/ DSM 20016/ *Lb. fermentum* type II	No Hit	NA	*Thiomargarita namibiensis* (bacteria)	0.264
Lb9	*Lb. reuteri* F275/ ATCC23272/DSM 20016 /Lb. fermentum type II	*Lb. xiangfangensis*	92%	*Lb. plantarum*	0.391
Lb10	*Lb. hilgardii* IOEB 9649	*Lb. brevis*	93%	*Lb. brevis*	0.479

BLAST: Basic Local Alignment Search Tool, RDP: Ribosomal Database Project, S-ab: Sequences Matching Lb: *Lactobacillus.* NA: Not Applicable.

Table 3.2 Identification of food *Lactobacillus* isolates using BLAST and RDP tools

Isolate Symbol	Isolation Source	Isolate Name in BLAST	Identity Percentage of the Forward Strand in BLAST	Identity Percentage of the Reverse Strand in BLAST	Isolate Name in RDP	S-ab Score of the Forward Strand in RDP	S-ab Score of the Reverse Strand in RDP
Lb15	Yogurt (P1)	*Lb. delbrueckii*	92%	98%	*Lb. delbrueckii*	0.635	0.920
Lb17	Yogurt (P1)	*Lb. delbrueckii*	92%	97%	*Lb. delbrueckii*	0.594	0.866
Lb18	Yogurt (P1)	*Lb. delbrueckii*	91%	96%	*Lb. delbrueckii*	0.621	0.806
Lb19	Yogurt (P1)	*Lb. delbrueckii*	87%	98%	*Lb. delbrueckii*	0.423	0.922
Lb20	Olive (P2)	*Lb. plantarum*	89%	97%	*Lb. plantarum*	0.495	0.843
Lb21	Olive (P2)	*Lb. plantarum*	85%	98%	*Lb. plantarum*	0.378	0.877
Lb22	Olive (P2)	*Lb. plantarum*	90%	99%	*Lb. plantarum*	0.493	0.989
Lb23	Olive (P3)	*Lb. plantarum*	87%	99%	*Lb. plantarum*	0.438	0.916
Lb24	Olive (P3)	*Lb. plantarum*	87%	99%	*Lb. plantarum*	0.447	0.984
Lb25	Olive (P3)	*Lb. plantarum*	84%	98%	*Lb. plantarum*	0.386	0.884

BLAST: Basic Local Alignment Search Tool, RDP: Ribosomal Database Project, S-ab: Sequence Matching Lb: *Lactobacillus.*

3.4.1.3 Identification of Pathogenic Bacterial Species

The taxonomic identity of the pathogenic isolates was confirmed by 16S rRNA gene sequencing. All the obtained sequences showed high similarities to the available databases with an identity percentage of 99% using BLAST tool, while the scores rate of the sequences matching in RDP was 0.971 - 0.978. Comparison of the 16S rRNA gene sequence of SA1 with existed sequences in each tool revealed a similarity to *Staphylococcus aureus*. Regarding the isolates SPA1 and SDG4, the sequences comparison showed that SPA1 matched with the species *Streptococcus pyogenes,* while SDG4 was identified as *Streptococcus dysgalactiae* subsp *equisimilis* (Table 3.3). Cultivation of these isolates on TSA supplemented with 5% v/v sheep blood revealed that both SA1 and SPA1 were non-haemolytic bacteria. These bacterial species which lack the ability to produce haemolysin, usually referred to as producing gamma haemolysis (γ-haemolysis). Whilst beta haemolysis (β-haemolysis) or complete haemolysis was

shown by SDG4, as this species completely lysed the red blood cells in the medium exhibiting a lighted (yellow) area around the colony.

Table 3.3 Identification of skin pathogenic isolates using BLAST and RDP tools.

Isolate Symbol	Isolation Source	Isolate Name	Isolate Name in BLAST	Identity Percentage in BLAST	Isolate Name in RDP	S-ab Score in RDP
SA1	Perineum	*S. aureus*	*S. aureus*	99%	*S. aureus subsp. aureus*	0.973
SPA1	Leg	*Streptococcus* GAS	*S.pyogenes* GAS	99%	*S. pyogenes GAS*	0.971
SDG4	Leg	*Streptococcus* GGS	*S. dysgalactiae* subsp. *equisimilis* GGS	99%	*S. dysgalactiae subsp. equisimilis*	0.978

BLAST: Basic Local Alignment Search Tool, RDP: Ribosomal Database Project, S-ab: Sequences Matching, S.: *Staphylococcus*, GAS: Group A Streptococci, GGS: Group G Streptococci.

3.4.2 Characterisation of Bacterial Species Draft Genomes

3.4.2.1 Characterisation of *Lactobacillus* Species Draft Genomes

The genetic analysis of the food isolated *Lactobacillus* species showed that each *Lb. delbrueckii* genomes (Lb15, Lb17, Lb18 and Lb19), had one gene encoding for hydrogen peroxide-inducible genes activator (*oxyR*). This gene is essential for the stimulation of a regulon of hydrogen peroxide-inducible genes. No bacteriocin-encoding genes were observed within these genomes. However, it was detected that *Lb. delbrueckii* genomes possessed genes encoding for the protein associated with the processing and transport of the bacteriocin lactococcin-G. These genes were lactococcin-G-processing and transport ATP-binding protein LagD. Two genes of this protein (*lagD_1* and *lagD_2*) were found in Lb15, Lb18 and Lb19, while Lb17 had only one gene (*lagD_1*). In terms of *Lb. plantarum* genomes (Lb20 - Lb25), two to three genes of *oxyR* were recognised in each isolate including, *oxyR_1*, *oxyR_2* and *oxyR_3*. Moreover, one gene encoding for the protein required for the regulation of fatty acids metabolism (*fadR*), was found in each of these isolates (Table 3.4). A number of proteins responsible for stress resistance were detected in all *Lactobacillus* genomes. For example, putative universal stress protein and acid shock protein. In addition to these, genes encoding for stress resistance proteins were also found such as, general stress protein A (*gspA*) in *Lb. delbrueckii* isolates, general stress protein 69 (*yhdN_1*, *yhdN_2*) and general stress protein 13 (*yugI_1*, *yugI_2*) in *Lb.*

plantarum isolates (data not shown). Information obtained from the assembly of *Lactobacillus* draft genomes is shown in Appendix 2.

3.4.2.2 Characterisation of Pathogenic Species Genomes

Numerous virulence factors were detected in the sequenced genomes of pathogenic bacterial species involved in the present study. Tables 3.5, 3.6 and 3.7 represents the factors associated with the virulence of SA1, SPA1 and SDG4, respectively. Proteins required for the biosynthesis of bacterial cell surface structures were detected in the bacterial genome such as pilin and capsular polysaccharide type 8 biosynthesis protein (cap8A). Pilin proteins belong to a class of fibrous proteins that are found in structures including pili and fimbriae, while cap8A protein is required for the biosynthesis of type 8 capsular polysaccharide. One or more genes encoding for capsular proteins were found within the genomes including, *capA*, *cap8A_1* and *cap8A_2*. The genome also contained genes encoding for some surface proteins which allow the bacterial adhesion to the host extracellular matrix (ECM) proteins, including clumping factors A (*clfA*) and B (*clfB*), fibrinogen-binding proteins (*fib_1*, *fib_2* and *fib_3*), fibronectin-binding protein A (*fnbA*, *fnbA_1* and *fnbA_2*), plasmin and fibronectin-binding protein A (*pfbA*), collagen-binding proteins (*cna*, *cna-1* and *cna_2*), a cluster of genes encoding to surface proteins which bind to non-specific extracellular matrix proteins and immunoglobulin G-binding protein A (*spa*, *spg* and *sbi*).

Several genes encoding for enzymes were observed in the genomes of the three pathogens such as, IgM protease (*ide*), serine protease (*splA*, *splB*, *splC* and *splF*), serine protease like proteins (*htrA*, *htrA_1*, *htrA_2*), cysteine proteases including staphopain A (*sspP*) and B (*sspB*) in the SA1 genome, and streptopain (*speB_1* and *speB_2*) in the SPA1 genome. Other enzymes were also produced by the pathogenic isolates like, coagulase, staphylokinase (*sak*) in SA1, Streptokinase C (*skc*, *skc_1* and *skc_2*) in both SPA1 and SDG4. Furthermore, all genomes contained genes encoding for lipase (*lipA_1*, *lipA_2* and *lip2*), phospholipase C (*hlb_1* and *hlb_2*), hyaluronate lyase and different urease subunits (*ureC*, *ureB* and *ureA*) in addition to a group of urease accessory proteins. Each of these enzymes has a specific role either in the degradation of the host tissue proteins or in the induction of the host immune system to facilitate the evasion and spread of the pathogen. A number of genes encoding for membrane damaging exotoxins were also identified in the bacterial genomes such as encoding for leucocidin and haemolysins including haemolysin A (*tylA*), haemolysin C (*tylC*), α-haemolysin (alpha, *hyl*), δ-

haemolysin (delta, *hld*) and γ-haemolysin (Gamma, *hlg*). Despite the existence of several genes encoding for different types of haemolysins in all three pathogenic genomes, the haemolytic phenotypes of these pathogens by the cultivation on blood agar showed no-haemolysis for both SA1 and SPA1, while SDG4 revealed a complete haemolysis (Section 3.4.1.3) suggesting that the availability of specific genes in bacterial genomes could not be considered as an indicator of their phenotypic features which they display on cultural media.

Additional genes of pyrogenic exotoxins were found in the genomes like, exotoxin H (*speH*), exotoxin C (*speC*), enterotoxin types A, C, D, E, G and H (*entA, entC, entD, entE, entG* and *entH*), and toxic shock syndrome toxin-1 (*tst_1* and *tst_2*). Moreover, three genes encoding for staphylococcal complement inhibitor (*scn_1*, *scn_2* and *scn_3*) were identified in SA1 genome. Lantibiotics encoding genes were detected in both SA1 and SPA1 genomes, whereas the genome of SDG4 had no lantibiotic genes. A gene of the lantibiotic streptin (*srtA*) was found in the two pathogens (SA1 and SPA1). Two more lantibiotic genes were distinguished in the SPA1 genome, these were salivaricin_A (salA_1) and gallidemin (gdmA). Genes encoding for the surface M protein were observed in both SPA1 and SDG4 genomes. Two genes of this surface protein were detected in the SPA1 genome including, *emm5_1* and *emm5_2*, while only one gene (*emm5*) was found in the genome of SDG4. One streptolysin O encoding gene (*slo*) was also recognised in each genome of SPA1 and SDG4.

Table 3.4 Detection of genes encoding for antibacterial compounds in food-isolated *Lactobacillus* genomes (Lb15 - Lb19: *Lb. delbrueckii*, Lb20 - Lb25: *Lb. plantarum*)

***Lactobacillus* Isolates**	**Classification**	**Product and Gene names**	**Locus_tag**	**Gene Position**
Lb15	Bacteriocin	Lactococcin-G-processing and transport ATP-binding protein LagD (lagD_1)	ADLPAJGC_01081	2473-899
	Bacteriocin	Lactococcin-G-processing and transport ATP-binding protein LagD (lagD_2)	ADLPAJGC_01416	2182-3759
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR)	ADLPAJGC_00023	25149-26054
Lb17	Bacteriocin	Lactococcin-G-processing and transport ATP-binding protein LagD (lagD_1)	JHNOMHIH_01080	2473-899
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR)	JHNOMHIH_00136	25210-26115
Lb18	Bacteriocin	Lactococcin-G-processing and transport ATP-binding protein LagD (lagD_1)	BKHDIGGB_01083	2473-899
	Bacteriocin	Lactococcin-G-processing and transport ATP-binding protein LagD (lagD_2)	BKHDIGGB_01418	2182-3759
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR)	BKHDIGGB_00136	24852-25757
Lb19	Bacteriocin	Lactococcin-G-processing and transport ATP-binding protein LagD (lagD_1)	BFEHAKFB_01072	2473-899
	Bacteriocin	Lactococcin-G-processing and transport ATP-binding protein LagD (lagD_2)	BFEHAKFB_01409	2182-3759
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR)	BFEHAKFB_00389	11663-12568
Lb20	Fatty acid	Fatty acid metabolism regulator protein (fadR)	FGPHMOCD_01212	11254-11835
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR_1)	FGPHMOCD_00433	122720-123637
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR_2)	FGPHMOCD_00567	105774-106667
Lb21	Fatty acid	Fatty acid metabolism regulator protein (fadR)	OJHGHBDA_01080	61068-60487
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR_1)	OJHGHBDA_00311	69061-69954
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR_2)	OJHGHBDA_01230	44578-43679
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR_3)	OJHGHBDA_02579	26680-25763
Lb22	Fatty acid	Fatty acid metabolism regulator protein (fadR)	CIABFNFE_01078	61068-60487
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR_1)	CIABFNFE_00310	69061-69954
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR_2)	CIABFNFE_01228	44578-43679
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR_3)	CIABFNFE_02576	26680-25763
Lb23	Fatty acid	Fatty acid metabolism regulator protein (fadR)	AOFENMBG_01081	61068-60487
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR_1)	AOFENMBG_00287	38866-37973
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR_2)	AOFENMBG_01231	44578-43679
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR_3)	AOFENMBG_02577	26680-25763
Lb24	Fatty acid	Fatty acid metabolism regulator protein (fadR)	MHIPKMEN_01025	6248-6829
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR_1)	MHIPKMEN_00286	38866-37973

	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR_2)	MHIPKMEN_01226	44578-43679
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR_3)	MHIPKMEN_02500	26632-25715
Lb25	Fatty acid	Fatty acid metabolism regulator protein (fadR)	GDBEJFHJ_00225	84329-83748
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR_1)	GDBEJFHJ_00545	25435-24518
	Hydrogen peroxide	Hydrogen peroxide-inducible genes activator (oxyR_2)	GDBEJFHJ_01662	55883-56776

Table 3.5 Detection of genes encoding for virulence factors in *Staphylococcus aureus* (SA1) genome.

Virulence Factor	Classification	Function	Product and Gene names	Locus_tag	Gene Position
Pilus / Fimbria	Adhesin	Allows bacterial adherence to host eukaryotic cells, helps in biofilm formation and enable horizontal genes transfer	Pilin	BFBEMJPD_02664	3230-1251
Capsule	Adhesin	Inhibits phagocytosis, promotes bacterial adherence to host cells and in prosthetic devices	Capsular polysaccharide type 8 biosynthesis protein cap8A (*cap8A_1*)	BFBEMJPD_00813	518-1186
			Capsular polysaccharide type 8 biosynthesis protein cap8A (*cap8A_2*)	BFBEMJPD_01590	40301-40963
Clumping factor	Adhesin	Allows bacterial adherence to fibrinogen and inhibits opsonophagocyrosis	Clumping factor A (*clfA*)	BFBEMJPD_00373	123782-126307
			Clumping factor B (*clfB*)	BFBEMJPD_01624	85878-88199
Fibrinogen-binding protein	Adhesin	Allows bacterial adherence to fibrinogen and inhibits opsonophagocyrosis	Fibrinogen-binding protein (*fib_1*)	BFBEMJPD_00650	120-527
			Fibrinogen-binding protein (*fib_2*)	BFBEMJPD_01753	106986-107315
Fibronectin-binding protein A	Adhesin	Allows bacterial adherence to host cells of another organism and facilitates the internalisation of bacteria to these cells	Fibronectin-binding protein A (*fnbA_1*)	BFBEMJPD_01308	24231-21358
			Fibronectin-binding protein A (*fnbA_2*)	BFBEMJPD_01309	27959-24912
			Plasmin and fibronectin-binding protein A (*pfbA*)	BFBEMJPD_01922	63812-62715
Collagen-binding protein	Adhesin	Allows bacterial adherence to collagen	Collagen adhesin (*cna*)	BFBEMJPD_01567	14793-18344
Extracellular matrix protein- binding protein	Adhesin	Allows bacterial adherence to different extracellular matrix proteins of the host cells	Extracellular matrix protein-binding protein emp (*ssp*)	BFBEMJPD_00375	128389-129411

			Extracellular matrix protein-binding protein emp (*emp*)	BFBEMJPD_00376	129741-130262
			Extracellular matrix-binding protein ebh (*ebh_1*)	BFBEMJPD_00495	7166-13735
			Extracellular matrix-binding protein ebh (*ebh_2*)	BFBEMJPD_01418	10-888
			Extracellular matrix-binding protein ebh (*ebh_3*)	BFBEMJPD_01768	5402-12718
			Extracellular matrix-binding protein ebh (*ebh_4*)	BFBEMJPD_02122	62900-76186
			Extracellular matrix-binding protein EbhA (*ebhA*)	BFBEMJPD_02123	76315-93789
Immunoglobulin G-binding protein	Adhesin	Prevents antibody opsonization and phagocytosis	Immunoglobulin G-binding protein A (*spa*)	BFBEMJPD_02316	37691-39085
			Immunoglobulin-binding protein sbi (*sbi*)	BFBEMJPD_00246	240715-242025
Immunoglobulin-binding protease	Extracellular enzyme	Cleaves the immunoglobulin and facilitates humoral immune evasion	IgM protease (*ide*)	BFBEMJPD_03312	772-50
Serine protease	Extracellular enzyme	Digests desmosome proteins and causes bullous disease	Serine protease Do-like HtrA (*htrA*)	BFBEMJPD_00523	51030-49756
			Serine protease HtrA-like protein	BFBEMJPD_02258	28806-26497
Staphopain / (Staphylococcal cysteine protease)	Extracellular enzyme	Degrades elastin and causes connective tissue destruction	Staphopain A (*sspP*)	BFBEMJPD_01911	52303-53469
			Staphopain B (*sspB*)	BFBEMJPD_02231	4437-5618
Staphylococcal pyrogenic exotoxin H	Pyrogenic exotoxin	Induces the proliferation of T-lymphocytes and stimulates the release of cytokines	Exotoxin type H (*speH*)	BFBEMJPD_00657	6660-5935
Staphylococcal pyrogenic exotoxin C	Pyrogenic exotoxin	Induces the proliferation of T-lymphocytes and stimulates the release of cytokines	Exotoxin type C (*speC*)	BFBEMJPD_01278	149292-149990
Staphylocoagulase	Extracellular enzyme		Staphylocoagulase	BFBEMJPD_00374	126528-128036

		Induces coagulation and converts soluble fibrinogen into fibrin which will protect bacteria from the immune system	Staphylocoagulase	BFBEMJPD_00887	93331-95301
Staphylokinase	Extracellular enzyme	Digests fibrin clots and activates fibrinolysis	Staphylokinase (*sak*)	BFBEMJPD_01955	96385-95894
Lipase	Extracellular enzyme	Cleaves sebum-derived triacylglycerols into fatty acids to enable the bacterial adherence, colonization and invasion through the skin barrier	Lipase 1 (*lipA_2*)	BFBEMJPD_01584	32875-34920
			Lipase 2 (*lip2*)	BFBEMJPD_01126	21242-23179
			Phospholipase C (*hlb_1*)	BFBEMJPD_01951	91960-92160
			Phospholipase C (*hlb_2*)	BFBEMJPD_02372	49365-48541
Hyaluronate lyase	Extracellular enzyme	Degrades hyaluronic acid, a major constituent of animal tissues	Hyaluronate lyase	BFBEMJPD_00036	38537-40963
			Hyaluronate lyase	BFBEMJPD_03019	78-341
Urease	Extracellular enzyme	Catalyses the hydrolysis of urea forming ammonia and carbon dioxide	Urease subunit alpha (*ureC*)	BFBEMJPD_00117	104974-106689
			Urease subunit beta (*ureB*)	BFBEMJPD_00116	104567-104977
			Urease subunit gamma (*ureA*)	BFBEMJPD_00115	104251-104553
			Urease accessory protein UreD (*ureD1*)	BFBEMJPD_00121	108463-109299
			Urease accessory protein UreE (*ureE*)	BFBEMJPD_00118	106702-107154
			Urease accessory protein UreF (ureF)	BFBEMJPD_00119	107147-107836
			Urease accessory protein UreG (*ureG*)	BFBEMJPD_00120	107849-108463
Haemolysin			Haemolysin A (*tlyA*)	BFBEMJPD_02936	139-669

	Membrane damaging exotoxin	Causes lysis of white and red blood cells by disruption of the cell membrane	Haemolysin C (*tlyC*)	BFBEMJPD_00410	154466-155506
			Alpha-haemolysin (*hly*)	BFBEMJPD_00654	3730-2771
			Delta-haemolysin (*hld*)	BFBEMJPD_02355	30459-30593
			Gamma-haemolysin component A (*hlgA*)	BFBEMJPD_00247	242480-243445
			Gamma-haemolysin component B (*hlgB*)	BFBEMJPD_00249	244962-245939
			Gamma-haemolysin component C (*hlgC_1*)	BFBEMJPD_00248	244013-244960
			Gamma-haemolysin component C (*hlgC_2*)	BFBEMJPD_02067	2905-3843
Leukocidin	Membrane-damaging exotoxin	Causes lysis of white blood cells by disruption of the cell membrane	putative leukocidin-like protein 1	BFBEMJPD_02371	47287-48303
			putative leukocidin-like protein 2	BFBEMJPD_02370	46213-47265
			Leucotoxin LukDv (*lukDv*)	BFBEMJPD_02068	3845-4822
Enterotoxin	Pyrogenic exotoxin	Causes food poisoning	Enterotoxin type A (*entA_1*)	BFBEMJPD_00638	180308-181165
			Enterotoxin type A (*entA_2*)	BFBEMJPD_02449	35338-36066
			Enterotoxin type A (*entA_3*)	BFBEMJPD_02451	37029-37784
			Enterotoxin type C-3 (*entC3*)	BFBEMJPD_02450	36220-36990
			Enterotoxin type D (*entD_1*)	BFBEMJPD_02447	33539-34303
			Enterotoxin type D (*entD_2*)	BFBEMJPD_02448	34584-35303
			Enterotoxin type G (*entG*)	BFBEMJPD_02452	38067-38843
Toxic shock syndrome toxin-1	Pyrogenic exotoxin	Causes toxic shock syndrome (TSS)	Toxic shock syndrome toxin-1 (tst_1)	BFBEMJPD_01273	143757-144437

			Toxic shock syndrome toxin-1 (tst_2)	BFBEMJPD_01279	150357-151040
Staphylococcal complement inhibitor	Secreted protein	Blocks the complement fixation and opsonins recognition	Staphylococcal complement inhibitor (*scn_1*)	BFBEMJPD_00651	679-1029
			Staphylococcal complement inhibitor (*scn_2*)	BFBEMJPD_00886	93141-92797
			Staphylococcal complement inhibitor (*scn_3*)	BFBEMJPD_01953	93685-93335
Lantibiotic	Bacteriocins	Inhibits the growth of other clinical Gram-positive pathogens	Lantibiotic streptin (*srtA*)	BFBEMJPD_03073	419-559

Table 3.6 Detection of genes encoding for virulence factors in *Streptococcus pyogenes* (SPA1) genome

Virulence Factor	Classification	Function	Product and Gene names	Locus_tag	Gene Position
Pilus / Fimbria	Adhesin	Allows bacterial adherence to host eukaryotic cells, helps in biofilm formation and enable horizontal genes transfer	Pilin	MMFMANDF_01192	61287-60259
			Pilin	MMFMANDF_01194	64087-61823
Capsule	Adhesin	Inhibits phagocytosis, promotes bacterial adherence to host cells and in prosthetic devices	Capsule biosynthesis protein CapA (*capA*)	MMFMANDF_00255	51666-52958
			Capsular polysaccharide type 8 biosynthesis protein cap8A (*cap8A_1*)	MMFMANDF_01853	19218-19880
			Capsular polysaccharide type 8 biosynthesis protein cap8A (*cap8A_2*)	MMFMANDF_03647	1119-451
Clumping factor A	Adhesin	Allows bacterial adherence to fibrinogen and inhibits opsonophagocyrosis	Clumping factor A (*clfA*)	MMFMANDF_04391	23-418
M protein	Adhesin	Mediates adherence to epidermal keratinocytes and protects the bacteria from phagocytosis	M protein, serotype 5 (*emm5_1*)	MMFMANDF_00387	189886-190851
			M protein, serotype 5 (*emm5_2*)	MMFMANDF_00389	193482-194288
Fibrinogen-binding protein	Adhesin	Allows bacterial adherence to fibrinogen and inhibits opsonophagocyrosis	Fibrinogen-binding protein (*fib_1*)	MMFMANDF_03172	787-1104
			Fibrinogen-binding protein (*fib_2*)	MMFMANDF_03175	3310-3807
			Fibrinogen-binding protein (*fib_3*)	MMFMANDF_04313	610-347

Fibronectin-binding protein A	Adhesin	Allows bacterial adherence to host cells of another organism and facilitates the internalisation of bacteria to these cells	Fibronectin-binding protein A (*fnbA*)	MMFMANDF_03597	154-669
Collagen-binding protein	Adhesin	Allows bacterial adherence to collagen	Collagen adhesin (*cna_1*)	MMFMANDF_01188	57166-53684
			Collagen adhesin (*cna_2*)	MMFMANDF_02304	3786-1357
Extracellular matrix protein- binding protein	Adhesin	Allows bacterial adherence to different extracellular matrix proteins of the host cells	Extracellular matrix protein-binding protein emp (*emp*)	MMFMANDF_03145	4932-4411
			Extracellular matrix-binding protein ebh (*ebh_1*)	MMFMANDF_01577	11521-17430
			Extracellular matrix-binding protein ebh (*ebh_2*)	MMFMANDF_03905	20-1039
			Extracellular matrix-binding protein ebh (*ebh_3*)	MMFMANDF_04346	404-36
			Extracellular matrix-binding protein EbhA (*ebhA_1*)	MMFMANDF_03018	72-1289
			Extracellular matrix-binding protein EbhA (*ebhA_2*)	MMFMANDF_03019	1347-3353
Immunoglobulin G-binding protein	Adhesin	Prevents antibody opsonization and phagocytosis	Immunoglobulin G-binding protein A (*spa*)	MMFMANDF_02115	13688-13503
			Immunoglobulin G-binding protein G (*spg*)	MMFMANDF_01617	15900-15163
			Immunoglobulin G-binding protein H	MMFMANDF_00717	126825-127868
			Immunoglobulin-binding protein sbi (*sbi*)	MMFMANDF_02037	6479-7792

Immunoglobulin-binding protease	Extracellular enzyme	Cleaves the immunoglobulin and facilitates humoral immune evasion	IgM protease (*ide*)	MMFMANDF_00289	88596-87571
Serine protease	Extracellular enzyme	Digests desmosome proteins and causes bullous disease	Serine protease HtrA-like protein	MMFMANDF_03663	3098-1797
			Serine protease Do-like HtrA (*htrA_1*)	MMFMANDF_01303	68368-69591
			Serine protease Do-like HtrA (*htrA_2*)	MMFMANDF_03079	2238-964
			Serine protease SplA (*splA*)	MMFMANDF_03941	402-10
			Serine protease SplC (*spIC*)	MMFMANDF_04155	128-643
			Serine protease SplF (*spIF*)	MMFMANDF_04333	476-252
Streptopain / (Streptococcal cysteine protease) / (Streptococcal pyrogenic exotoxin B)	Extracellular enzyme	Degrades elastin and causes connective tissue destruction	Streptopain (*speB_1*)	MMFMANDF_00674	75117-76313
			Streptopain (*speB_2*)	MMFMANDF_00675	76315-76620
Streptococcal pyrogenic exotoxin C	Pyrogenic exotoxin	Induces the proliferation of T-lymphocytes and stimulates the release of cytokines	Exotoxin type C (*speC_1*)	MMFMANDF_00288	87395-87186
			Exotoxin type C (*speC_2*)	MMFMANDF_00704	114277-113585
			Exotoxin type C (*speC_3*)	MMFMANDF_01944	11311-12015
			Exotoxin type C (*speC_4*)	MMFMANDF_03524	1193-1888
Streptococcal pyrogenic exotoxin H	Pyrogenic exotoxin	Induces the proliferation of T-lymphocytes and stimulates the release of cytokines	Exotoxin type H (*speH*)	MMFMANDF_04105	256-981
Streptokinase	Extracellular enzyme	Digests fibrin clots and activates fibrinolysis	Streptokinase C (*skc*)	MMFMANDF_00720	132217-130895
Lipase	Extracellular enzyme	Cleaves sebum-derived triacylglycerols into fatty acids to enable the bacterial	Lipase 1 (*lipA_1*)	MMFMANDF_01847	11739-13784
			Lipase 2 (*lip2*)	MMFMANDF_02739	2292-358

		adherence, colonization and invasion through the skin barrier	Phospholipase C (*hlb_1*)	MMFMANDF_02230	6495-6695
			Phospholipase C (*hlb_2*)	MMFMANDF_03316	920-96
Hyaluronate lyase	Extracellular enzyme	Degrades hyaluronic acid, a major constituent of animal tissues	Hyaluronate lyase	MMFMANDF_00374	177710-175293
			Hyaluronate lyase	MMFMANDF_02806	7628-5679
Urease	Extracellular enzyme	Catalyses the hydrolysis of urea forming ammonia and carbon dioxide	Urease subunit alpha (*ureC*)	MMFMANDF_03678	53-853
			Urease subunit beta (*ureB*)	MMFMANDF_03522	3225-3635
			Urease subunit gamma (*ureA*)	MMFMANDF_03521	2909-3211
			Urease accessory protein UreE (*ureE*)	MMFMANDF_03679	866-1318
			Urease accessory protein UreF (*ureF*)	MMFMANDF_03680	1311-2000
			Urease accessory protein UreG (*ureG*)	MMFMANDF_03681	2013-2627
Streptolysin O	Membrane-damaging exotoxin	Causes haemolysis of red blood cells (beta-haemolysis)	Streptolysin O (*slo*)	MMFMANDF_01162	27373-25658
Haemolysin	Membrane-damaging exotoxin	Causes lysis of white and red blood cells by disruption of the cell membrane	Haemolysin A (*tlyA*)	MMFMANDF_01042	10599-9772
			Haemolysin C (*tlyC*)	MMFMANDF_03917	2090-1050
			Delta-haemolysin (*hld*)	MMFMANDF_02694	6615-6481
			Gamma-haemolysin component A (*hlgA*)	MMFMANDF_02039	8292-9257
			Gamma-haemolysin component B (*hlgB*)	MMFMANDF_02041	10774-11751

			Gamma-haemolysin component C (*hlgC*)	MMFMANDF_02040	9825-10772
Leukocidin	Membrane-damaging exotoxin	Causes lysis of white blood cells by disruption of the cell membrane	Putative leukocidin-like protein 1	MMFMANDF_03686	1072-56
			Putative leukocidin-like protein 2	MMFMANDF_03687	2146-1094
			Leucotoxin LukEv (*lukEv*)	MMFMANDF_04117	951-16
			Leucotoxin LukDv (*lukDv*)	MMFMANDF_04351	178-513
Enterotoxin	Pyrogenic exotoxin	Causes food poisoning	Enterotoxin type A (*entA_1*)	MMFMANDF_02238	12598-11870
			Enterotoxin type A (*entA_2*)	MMFMANDF_02860	183-872
			Enterotoxin type A (*entA_3*)	MMFMANDF_04183	24-449
			Enterotoxin type A (*entA_4*)	MMFMANDF_04184	473-1150
			Enterotoxin type H (*entH*)	MMFMANDF_02298	8005-8730
Toxic shock syndrome toxin-1	Pyrogenic exotoxin	Causes toxic shock syndrome (TSS)	Toxic shock syndrome toxin-1 (*tst_1*)	MMFMANDF_03193	326-1009
			Toxic shock syndrome toxin-1 (*tst_2*)	MMFMANDF_03641	1425-745
Lantibiotic	Bacteriocins	Inhibits the growth of other clinical Gram-positive pathogens	Lantibiotic streptin (*srtA*)	MMFMANDF_00011	6543-6683
			Lantibiotic salivaricin-A (*salA_1*)	MMFMANDF_00763	179080-179226
			Lantibiotic gallidermin (*gdmA*)	MMFMANDF_04180	797-940

Table 3.7 Detection of genes encoding for virulence factors in *Streptococcus dysgalactiae* subsp *equisimilis* (SDG4) genome

Virulence Factor	Classification	Function	Product and Gene names	Locus_tag	Gene Position
Pilus / Fimbria	Adhesin	Allows bacterial adherence to host eukaryotic cells, helps in biofilm formation and enable horizontal genes transfer	Pilin	JHIEOLAN_00966	86884-88389
			Pilin	JHIEOLAN_00968	88952-89974
Capsule	Adhesin	Inhibits phagocytosis, promotes bacterial adherence to host cells and in prosthetic devices	Capsule biosynthesis protein CapA (*capA*)	JHIEOLAN_00883	101579-102772
			Capsular polysaccharide type 8 biosynthesis protein cap8A (*cap8A*)	JHIEOLAN_02123	2752-2090
M protein	Adhesin	Mediates adherence to epidermal keratinocytes Protects the bacteria from phagocytosis	M protein, serotype 5 (*emm5*)	JHIEOLAN_00434	48039-46225
Fibrinogen-binding protein	Adhesin	Allows bacterial adherence to fibrinogen and inhibits opsonophagocyrosis	Fibrinogen-binding protein	JHIEOLAN_03283	403-26
Fibronectin-binding protein A	Adhesin	Allows bacterial adherence to host cells of another organism and facilitates the internalisation of bacteria to these cells	Fibronectin-binding protein A (*fnbA_1*)	JHIEOLAN_02248	105-938
			Fibronectin-binding protein A (*fnbA_2*)	JHIEOLAN_03017	603-19
Collagen-binding protein	Adhesin	Allows bacterial adherence to collagen	Collagen adhesin (*cna*)	JHIEOLAN_02875	10-303
Extracellular matrix protein- binding protein	Adhesin	Allows bacterial adherence to different extracellular matrix proteins of the host cells	Extracellular matrix-binding protein ebh (*ebh*)	JHIEOLAN_02465	296-78
			Extracellular matrix-binding protein EbhA (*ebhA*)	JHIEOLAN_03299	91-345

Immunoglobulin G-binding protein	Adhesin	Prevents antibody opsonization and phagocytosis	Immunoglobulin G-binding protein A (*spa*)	JHIEOLAN_02983	366-181
			Immunoglobulin G-binding protein G (*spg*)	JHIEOLAN_00126	137962-139308
Serine protease	Extracellular enzyme		Serine protease Do-like HtrA (*htrA*)	JHIEOLAN_01912	1071-2294
			Serine protease SplA (*splA*)	JHIEOLAN_02707	5-181
			Serine protease SplB (*splB*)	JHIEOLAN_02708	306-788
Streptococcal pyrogenic exotoxin C	Pyrogenic exotoxin	Induces the proliferation of T-lymphocytes and stimulates the release of cytokines	Exotoxin type C (*speC_1*)	JHIEOLAN_01750	26143-26847
			Exotoxin type C (*speC_2*)	JHIEOLAN_02352	278-973
Streptokinase	Extracellular enzyme	Digests fibrin clots and activates fibrinolysis	Streptokinase C (*skc_1*)	JHIEOLAN_00430	41588-42910
			Streptokinase C (*skc_2*)	JHIEOLAN_01054	70501-69557
Lipase	Extracellular enzyme	Cleaves sebum-derived triacylglycerols into fatty acids to enable the bacterial adherence, colonization and invasion through the skin barrier	Lipase 1 (*lipA*)	JHIEOLAN_02200	974-1651
			Phospholipase C (*hlb*)	JHIEOLAN_02661	21-404
Hyaluronate lyase	Extracellular enzyme	Degrade hyaluronic acid, a major constituent of animal tissues	Hyaluronate lyase (*hylB*)	JHIEOLAN_01830	19992-23195
Urease	Extracellular enzyme	Catalyses the hydrolysis of urea forming ammonia and carbon dioxide	Urease subunit alpha (*ureC*)	JHIEOLAN_02584	978-685
			Urease accessory protein UreD (*ureD*)	JHIEOLAN_02232	500-201
			Urease accessory protein UreD (*ureD1*)	JHIEOLAN_02233	971-519
			Urease accessory protein UreE (*ureE*)	JHIEOLAN_02583	672-220

			Urease accessory protein UreF (*ureF*)	JHIEOLAN_02235	1732-1598
			Urease accessory protein UreG (*ureG*)	JHIEOLAN_02234	1585-971
Streptolysin O	Membrane-damaging exotoxin	Causes haemolysis of red blood cells (beta-haemolysis)	Streptolysin O (*slo*)	JHIEOLAN_01908	19457-21172
Haemolysin	Membrane-damaging exotoxin	Causes lysis of white and red blood cells by disruption of the cell membrane	Haemolysin A (*tlyA*)	JHIEOLAN_00042	43065-43892
			Gamma-haemolysin component A (*hlgA*)	JHIEOLAN_02525	1012-317
			Gamma-haemolysin component B (*hlgB*)	JHIEOLAN_02145	1061-84
			Gamma-haemolysin component C (*hlgC*)	JHIEOLAN_02146	2010-1063
Leukocidin	Membrane-damaging exotoxin	Causes lysis of white blood cells by disruption of the cell membrane	Putative leukocidin-like protein 1	JHIEOLAN_02662	869-642
			Putative leukocidin-like protein 2	JHIEOLAN_02717	435 -88
Enterotoxin	Pyrogenic exotoxin	Causes food poisoning	Enterotoxin type A (*entA*)	JHIEOLAN_02710	783-412
			Enterotoxin type E (*entE*)	JHIEOLAN_03242	67-255
			Enterotoxin type H (*entH*)	JHIEOLAN_02141	1286-2011
Toxic shock syndrome toxin-1	Pyrogenic exotoxin	Causes toxic shock syndrome (TSS)	Toxic shock syndrome toxin-1 (*tst*)	JHIEOLAN_02619	912-229

3.5 Discussion

3.5.1 Isolation and Identification of Lactic Acid Bacterial Species by 16S rRNA gene sequencing

L. delbrueckii and *Lb. plantarum* are the two bacterial species which were identified in this study using 16S rRNA gene sequencing. Yogurt was the isolation source of the first species and two olive types were used for the isolation of the second species. Novel strains originated from the olive microbiota have been isolated and characterised by several investigations. These strains were used as starter cultures in table olive processing and were well known for their probiotic features (Bevilacqua *et al.* 2010; Peres *et al.* 2012; Bautista-Gallego *et al.* 2013; Botta *et al.* 2014). The findings of these studies were promising indicating that olives are potential sources of probiotic candidates. Probiotic properties of table olive products are predicted to boost their important nutritional values. These products are also considered as a source of fibre, organic acids, vitamins and minerals (Argyri *et al.* 2013). Shafighi and colleagues indicated the significant importance of olives as one of the plants which have valuable probiotic content (Shafighi *et al.* 2012). Several studies determined the important compounds of olives, nevertheless, few studies have presented their probiotic features (Rodríguez *et al.* 2008).

Since *Lb. plantarum* has the ability to inhabit a broad range of nutritional environments, isolation of various strains has been conducted from fermented milk products, vegetables and plants (Cai *et al.* 1999). For instance, *Lb. plantarum* SK151, *Lb. plantarum* K25 and *Lb. plantarum* LL441 isolated from Kimchi, Tibetan Kefir and dairy products, respectively (Amoranto *et al.* 2018; Flórez and Mayo 2018; Jiang *et al.* 2018). The large genome size of *Lb. plantarum* and the high numbers of accessory genes, indicate the species adaptation ability to variant ecological conditions (Claesson *et al.* 2007). *Lb. plantarum* can naturally inhabit the human gastrointestinal tract and oral cavity, thus, this species was identified for its various probiotic applications (Molin *et al.* 1993; Zhang *et al.* 2012). Moreover, *Lb. plantarum* has antibacterial efficiency against Gram-positive pathogens such as, *S. aureus* and *L. monocytogenes* and also against Gram-negative bacteria like, *E. coli*, *Klebsiella*, *Yersinia* and *Salmonella typhimurium* (Silva *et al.* 2018; Spangler *et al.* 2019).

The original isolation source of *L. delbrueckii* ssp. *bulgaricus* (*L. bulgaricus*) was the Bulgarian yogurt (Orla-Jensen 1919, unpublished work) and it is used in the yogurt industry with the cooperation of *Streptococcus thermophilus*. Further, this species is considered as a safe

probiotic with multiple valuable characteristics (Adolfsson *et al.* 2004; Claesson *et al.* 2007). The collaboration of *L. bulgaricus* and *S. thermophilus* lead to an increase in the acidity of milk during fermentation (Hols *et al.* 2005).

3.5.2 Characterization of Bacterial Species Draft Genomes

3.5.2.1 Genomic Analysis and Characterization of *Lactobacillus* Species

Regarding *Lb. delbrueckii* isolates, genes associated with the transport and processing of lactococcin-G bacteriocin were observed in their genomes. Bacteriocins are small ribosomally synthesized antimicrobial peptides which are produced by bacteria and have an antibacterial activity against other closely related bacterial species (Cotter *et al.* 2005b). Lactococcin-G consists of two different peptides α and β; thus, it is classified as a two-peptides bacteriocin. The complementary action of both peptides in almost similar amounts is required for the antibacterial effect (Moll *et al.* 1996; Anderssen *et al.* 1998). It was found by Khalaf and associates that bacteriocins reveal their antibacterial effect against pathogens by damaging the outer membrane integrity, which is caused as a result of the increased permeability and the outflow of different ions into cells leading to death (Khalaf *et al.* 2016). In the presence of membrane structures, the two lactococcin G peptides co-operate with each other and form amphiphilic α-helices which are inserted into the membrane of the target cell creating potassium-selective channels (Moll *et al.* 1996; Hauge *et al.* 1998). It was declared by Ehrmann and co-worker that the bacteriocin produced by LAB may play a role in the progress of the colonisation process within different environments such as fermented chesses, vegetables and sausages, fermenting olives and wine, as well as the human gastrointestinal tract and saliva (Ehrmann *et al.* 2000). Bacteriocin producing lactobacilli have the ability to compete with other bacterial species available in the same habitat. Therefore, bacteriocins can also be utilised by the produced bacteria as a significant mechanism to eliminate the pathogens in fermented foods and in the GI tract as well. Furthermore, the bacteriocin biosynthesis is regarded as a desirable feature for the selected strain included in food products (Goh and Klaenhammer 2009). Thus, this characteristic attracts more attention on the application of LAB as preservatives in food industry (Cotter *et al.* 2005b), such as nisin-producing strains of *Lactococcus lactis* which produce the bacteriocin nisin A. These strains are used as protective cultures in several fermented dairy and vegetables because of their broad commercial advantages (Ross *et al.* 1999; Meijerink *et al.* 2010; Silva *et al.* 2018). It has been acknowledged by van de Guchte and colleagues that several distinctive features were recognised in the genome sequence of *L. delbrueckii* ssp. *bulgaricus* (*L. bulgaricus*) showing an

evidence of a rapid evolution phase of the genome. *L. delbrueckii* is considered as atypical species among the lactobacilli of the acidophilus complex due to several reasons. For example, a difference in GC content was observed in the genome sequence of this species. Furthermore, a large number of pseudogenes was detected in the *L. bulgaricus* genome suggesting its ability to adapt to the dairy environment (van de Guchte *et al.* 2006).

In terms of *Lb. plantarum* genomes, genes encoding for the regulator proteins essential in fatty acids metabolism, were detected. Many investigations reported the antibacterial effect of fatty acids, such as 3-hydroxy fatty acid produced by *Lb. plantarum* MiLAB 14 strain (Sjögren *et al.* 2003; Ogawa *et al.* 2005). It was declared by Bergsson and co-workers that fatty acids penetrated into pathogenic cells can bind to the bacterial plasma membrane and disrupt the permeability and stability of the membrane resulting in the bacteriostatic activity (Bergsson *et al.* 2001). Mao and colleagues recognised the fatty acids and organic acids as the compounds responsible for the antibacterial activity of *Lb. plantarum* DY-6. This research group detected that the growth inhibition of pathogens is achieved by the destruction of cell membrane stability (Mao *et al.* 2020). No genes encoding for bacteriocins were found in any of *Lb. plantarum* isolates involved in the present study. These results were in disagreement with Omar and colleagues who stated that many *Lb. plantarum* strains isolated from various niches have the ability to produce several bacteriocins. This research groups declared that an individual strain usually encodes for more than one bacteriocin within a locus existed on the chromosomal DNA (Omar *et al.* 2008). For example, the genetic determinants of three bacteriocins were detected in *Lb. plantarum* WCFS1, i.e. plantaricin N and the two peptides bacteriocins including plantaricin EF and plantaricin JK (Kleerebezem *et al.* 2003). Several investigations reported the presence of genes encoding for two bacteriocins, plantaricin W and plantaricin S in *Lb. plantarum* strains isolated from fermented wine and olives, respectively (Jiménez-Díaz *et al.* 1993; Holo *et al.* 2001). The bactericidal mechanism of plantaricin can be achieved by the creation of pores in the cell membrane of the indicator strains, resulting in membrane disruption and outflow of large molecules (Todorov 2009). The first sequenced *Lactobacillus* genome was *L. plantarum* WCFS1 (Kleerebezem *et al.* 2003). Devi and Halami reported the genetic diversity of *Lb. plantarum* strains with regards to the size of genome, number of proteins and variability in plantaricin encoding locus (Devi and Halami 2019). Li and colleagues stated that the genome size of *Lb. plantarum* differs from 3.0 to 3.3 Mb (Li *et al.* 2015).

Overall, both *Lb. plantarum* and *Lb. delbrueckii* genomes involved in this study had genes encoding for antibacterial compounds. In addition, these genomes included several genes

of the hydrogen peroxide sensor, which activates the expression of a regulon of hydrogen peroxide-inducible genes. Hydrogen peroxide produced during the fermentation process was known for its antibacterial efficiency against pathogenic bacteria (Castillo *et al.* 2002; Atanasova *et al.* 2014). It was acknowledged by several studies that ethnic fermented foods of Sikkim in India consist of a number of *Lactobacillus* species that possess antimicrobial activities (Tamang *et al.* 2008; Tamang and Tamang 2009). Furthermore, several probiotic genes were also observed in all *Lactobacillus* genomes analysed in the present study. These genes help the bacterial species to tolerate different types of stress caused by the exposure to heat, acid and bile, resulting in increasing the bacterial capacity to survive *in vitro* ecological stresses and *in vivo* human gastrointestinal tract circumstances (Varankovich *et al.* 2015; Goel *et al.* 2020). Labeer and colleagues stated that probiotic *Lactobacillus* reveals probiotic features such as adherence capability and stress response (Lebeer *et al.* 2008). Moreover, this bacterium has several adaptation characteristics which enable it to interact with other microbes and modulate the host immune response (Llewellyn and Foey 2017).

3.5.2.2 Genomic Analysis and Characterization of Pathogenic Species Genomes

A number of genes encoding for adhesins were detected within genomes of the pathogenic bacteria involved in the present study like, fibronectin-binding protein A (*fnbA*), fibrinogen-binding proteins (*fib*), collagen-binding proteins (*cna*) and additional extracellular matrix-binding proteins (*ssp*, *emp*, *ebh*). Some of these surface proteins have additional functions for the pathogen rather than the bacterial adhesion. It was stated by Foster and Geoghegan (2015) that fibronectin-binding proteins (FnBPs) allow the bacterial invasion of epithelial and endothelial cells, furthermore, FnBPs help in biofilm formation. Both FnBPs and collagen-binding proteins belong to the MSCRAMMs group, and they have well known functions in the bacterial adherence to host proteins and bacterial invasion into host cells (Patti *et al.* 1994; Hauck and Ohlsen 2006). Both extracellular matrix-binding proteins (Emps) and fibrinogen-binding proteins are anchorless proteins belonging to the SERAMs group (Hussain *et al.* 2001). Emps were initially known by their aptitude to bind to different extracellular matrix proteins and no specific protein is recognised by Emps (Hussain *et al.* 2001; Geraci *et al.* 2017). Some of these proteins have roles in invasion of eukaryotic cells and modulation of immune system, however, the importance of other proteins within this group during infection is not clear (Hussain *et al.* 2001; Harraghy *et al.* 2005). It has been declared by Bur and collaborators that the adhesion ability of Emps to skin ECM was higher than that shown towards bone or cartilge, indicating the Emps play a role in wound infections (Bur *et al.* 2013). Emps are required by *S. aureus* for

bacterial cells accumulation which is necessary to form an abscess and to persist within the host tissues (Cheng *et al.* 2009).

Pilin protein genes was detected in all the sequenced genomes. Several bacterial fibrous structures are formed by the Emps such as the pili and fibres which are usually existed in bacterial adhesins (Klemm and Schembri 2000). The adherence of *S. aureus* to catheters or foreign materials could be enabled by the fibre-like structure made of Emps leading to form an abscess and biofilm (Cheng *et al.* 2009). Fimbriae adhesins are mostly found in Gram- negative bacteria, nevertheless Gram-positive bacteria can also have many of these structures (Kline *et al.* 2009). It was stated by Piepenbrink and Sundberg that pilin proteins play multiple roles, as they can assist the bacteria to form a biofilm, transfer the genes horizontally and adhere to eukaryotic cells (Piepenbrink and Sundberg 2016).

SA1 genome had two genes encoded for clumping factor A (*clfA*) and clumping factor B (*clfB*), while SPA1 had one gene of *clfA* and no clumping factors genes were observed in SDG4 genome. The clumping factor (clf) is a cell wall surface-expressed protein that is considered as an adhesin molecule. Clf stimulates bacterial adhesion to the blood plasma protein fibrinogen. This binding results in a bacterial cell surface covered with this host protein and leads to the reduction of opsonophagocytosis (Foster 2005). It was mentioned by McDevitt and team-mates that clf plays a role in clumping ability of *S. aureus* incubated in serum (McDevitt *et al.* 1994). Bacterial genomes of the three pathogens possessed genes encoding for essential proteins required for the polysaccharide capsule synthesis. Capsular polysaccharides are usually produced by invasive diseases causing bacteria. Encapsulated bacteria can resist phagocytosis and are able to persist in the blood-stream of infected hosts. Therefore, capsular polysaccharides can increase the virulence of pathogenic bacteria (Portolés *et al.* 2001; Li *et al.* 2014). It has been reported by Cocchiaro and colleagues that approximately 80% of *S. aureus* isolates collected from patients are capsule forming bacteria (Cocchiaro *et al.* 2006). Expressed capsular polysaccharides (CP) of many clinical isolates of *S. aureus* are either serotype 5 CP (CP5) or serotype 8 CP (CP8) (Hochkeppel *et al.* 1987). In *S. pyogenes*, the major component of the bacterial capsule is hyaluronic acid. Since the capsule contributes in the intercellular adherence between bacteria, it allows the bacterial cells to form a biofilm (Cho and Caparon 2005; Haas *et al.* 2015).

Genes encoding for proteins with the ability to bind to immunoglobulin G (immunoglobulin G-binding proteins) were observed in the bacterial genomes including IgG-

binding protein A (*spa*) (which are found in all the three pathogens), IgG-binding protein G (*spg*), IgG-binding protein H and Ig-binding protein sbi (*sbi*). Binding of these proteins to IgG leads to IgG coating cells, this IgG molecule lacks its function as an opsonin or as an initiator of complement fixation (Smith *et al.* 2011).

Pathogenic bacterial species involved in this study possess secreted proteolytic enzymes, including serine proteases, cysteine proteases and serine-like proteases. In terms of serine protease enzyme, SPA1 genome had genes encoding for three types of this enzyme, SplA (*splA*), SplC (*splC*) and SplF (*splF*), while SplA (*splA*) and SplB (*splB*) were detected in SDG4 genome. No serine protease genes were found in the genome of SA1. However, SA1 genome possessed genes encoding for serine-like proteases HtrA (*htrA*) and putative CtpA-like serine protease which are homologues of serine protease. These genes were also observed in SPA1 and SDG4 genomes. Regarding the cysteine protease enzyme, this is called staphopain in SA1 and was existed in the genome as staphopain A (*sspP*) and staphopain B (*sspB*). In SPA1, this enzyme is termed streptopain or streptococcal pyrogenic exotoxin B and was detected as two genes including *speB_1* and *speB_2*. One gene encoding for putative cysteine protease YraA (*yraA*) was observed in both SA1 and SPA1 genomes. Cysteine protease or streptopain enzyme was not found in SDG4 genome. The role of extracellular proteases during infection has been investigated by several research groups, nonetheless, the findings were inconsistent. A reduction in the virulence of *S. aureus* RN6390 was reported by Coulter and colleagues in three various animal models, after a mutation in serine protease (Coulter *et al.* 1998). Similar results were found by another study when the strain 8325-4 of *S. aureus* also revealed attenuated virulence in a murine skin abscess model, following mutations in different serine-like proteases (Shaw *et al.* 2004). Other studies showed opposite results about the function of extracellular proteases in the pathogenicity. Shaw and co-workers stated that the ability of *S. aureus* 8325-4 to form a skin abscess, has not been influenced by the mutation induced in staphopain A and B (Shaw *et al.* 2004). Furthermore, no reduced virulence was observed in murine model after the mutations of several proteases like staphopain A and B (Calander *et al.* 2004). Further to these general roles of secreted proteases, it has been demonstrated that these enzymes have the ability to degrade particular proteins of the host tissues. For instance, human fibrinogen and fibronectin can be cleaved by staphopain B produced by *S. aureus*, which may assist in the bacterial spread (Massimi *et al.* 2002). In addition, exoproteases are able to cleave the chains of all human immunoglobulin classes (Prokešová *et al.* 1992) and elastin (Potempa *et al.* 1988) resulting in facilitating the tissue invasion. Moreover, several studies reported that proteolytic enzymes play

a role in the immune response evasion and phagocytosis repression (Laarman *et al.* 2011; Laarman *et al.* 2012).

The coagulase enzyme is usually produced by *S. aureus*; therefore, it was found in SA1 genome as two genes. It plays a role in the initiation of clot formation in human plasma (Donabedian and Boyle 1998). This feature is well studied in *S. aureus* and it has been indicated as a significant strategy for virulence stimulation (Hendrix *et al.* 1983; Proctor *et al.* 1984). It has been reported by Donabedian and Boyle that *S. pyogenes* can also form clots when it is grown in nutrient-poor medium below 37ºC. Therefore, once there are inappropriate conditions for bacterial invasion, the bacteria may utilise this feature as an adaptive mechanism to survive in host tissues (Donabedian and Boyle 1998). While the production of staphylocoagulase is a mediator of clot formation in *S. aureus*, staphylokinase enzyme produced by the bacteria can promote clot lysis (Christner and Boyle 1996). The enzyme which is responsible for thrombolytic activity in *S. pyogenes* is streptokinase (Donabedian and Boyle 1998). One gene encoding for staphylokinase (*sak*) was found in SA1 genome. SPA1 had also one gene encoding for streptokinase C (*skc*), while two genes of this enzyme were detected in SDG4 genome including, *skc_1* and *skc_2*. Three genes encoding for staphylococcal complement inhibitors were observed in SA1 genome. Products expressed by these genes enable the human pathogens to evade the complement system by the blockage of complement fixation, obstruction of opsonin detection and stimulation of opsonin degradation (Rooijakkers and van Strijp 2007; Lambris *et al.* 2008). Furthermore, these proteins play a role in prevention of the WBCs inflow into the infected site (Foster 2005).

A group of exoproteins are also secreted by all pathogenic bacterial species included in the recent study, like exotoxins and enzymes, involving proteases, lipases, ureases and hyaluronate lyases. These proteins might play a major role in the conversion of host tissues into nutrients essential for the bacterial growth (Dinges *et al.* 2000). Lipase encoding genes were found in the bacterial genomes. Lipase 1 gene (*lipA*) was found in all the pathogenic bacteria, whereas both SA1 and SPA1 had lipase 2 gene (*lip2*) as well. This enzyme has the ability to catalyse both the hydrolysis and synthesis of ester bonds of triacylglycerols (TAG) (Stehr *et al.* 2003). The importance of extracellular lipases as microbial virulence factors have been increasingly indicated in human pathogenic bacteria (Jaeger *et al.* 1994). Lipids digestion for nutrient acquisition is the most important function of extracellular lipases for the bacteria. These enzymes could assist the bacterial growth within an environment which contains lipids as the unique source of carbon (Stehr *et al.* 2003). Longshaw and co-workers reported that the

extracellular lipolytic system revealed a supportive effect on the growth of *S. aureus* (Longshaw *et al.* 2000). Rollof and co-workers stated that the production of lipase in clinical strains of *S. aureus* isolated from deep infections was significantly higher than those derived from superficial sites, suggesting the possible importance of lipase for the bacterial nutrition and spread (Rollof *et al.* 1987). Lipases may also show a direct function affecting the bacterial virulence and the pathogenesis of staphylococcal skin diseases. Furthermore, the *in vitro* incubation of granulocytes with lipase resulted in low phagocytic killing of bacteria. This could be due to the damaged action of the enzyme on the immune cell surface structures (Rollof *et al.* 1988; Stehr *et al.* 2003). Lipases play another role in the promotion of the bacterial adherence to host tissues and/or adjacent cells (Stehr *et al.* 2003). In addition to the lipolytic effect of microbial lipases, a further phospholipolytic activity of lipase 2 was described by van Kampen and colleagues. This research group observed that lipase 2 produced by *Staphylococcus warneri* could cause host tissue degradation and cells lysis as a result of phospholipids cleavage, which are considered as the main constituent of cell membranes (van Kampen *et al.* 2001). Longshaw and co-workers declared that *S. epidermidis*, which is a skin inhabitant and an opportunistic pathogen, secretes two lipases during the infection. These lipases might support the bacterial growth and colonization by cleaving the sebum-derived TAG (Longshaw *et al.* 2000). Other genes encoding for phospholipase C (PLC) enzyme were detected in pathogenic bacterial genomes involving in the present study. Two genes of PLC (*hlb_1* and *hlb_2*) were recognised in both SA1 and SPA1, while SDG4 had only one gene (hlb). König and colleagues found out that the synergistic effect of lipase and phospholipase enzymes stimulated the production of the immune reactive substances from neutrophils and platelets. This may disturb the immune responses, which resulted in the initiation of tissue damage and induction of inflammatory processes (König *et al.* 1996).

Genes coding for the extracellular enzyme, hyaluronate lyase (HL), were also found in the three pathogenic species. Two genes encoding for this enzyme were found in each of SA1 and SPA1 genomes, while only one gene (*hylB*) existed in SDG4 genome. The enzyme has the ability to degrade the mucopolysaccharide hyaluronic acid (HA) which is the main constituent of human and animal connective tissues (Arvidson 1983). It was observed by Choudhuri and Chakrabarty that HL could be produced by 91.2% of *S. aureus* strains, indicating the role of this enzyme in the bacterial virulence (Choudhuri and Chakrabarty 1969). Makris and co-workers detected that HL was produced during the early to mid-exponential phases of *S. aureus* growth, compared to other staphylococcal exoproteins which are usually produced during late stages of

the bacterial infection. This observation supported the enzyme contribution in the first stages of infection (Makris *et al.* 2004). Hyaluronate lyase produced by streptococci has significant impacts on the pathogenesis. The damaged hyaluronic acid provides the bacterium with nutrients required for the growth and aids the bacterial dissemination into host tissues (Hynes and Walton 2000). *S. pyogenes* produces this enzyme to assist the bacterial growth and spread in to subcutaneous tissues (Starr and Engleberg 2006). Nevertheless, an inactive form of hyaluronate lyase was detected in the virulent strains of this species (Hynes *et al.* 2009). Since *S. pyogenes* synthesizes a capsule which is mainly formed of hyaluronic acid, expression of the functional enzyme in this bacterium could degrade the capsule, resulting in development of the bacterial exposure to the host immune system. Thus, it was suggested by Hynes and colleagues that hyaluronate lyase functions as an anti-virulence factor in *S. pyogenes* (Hynes *et al.* 2009).

Genes encoding for urease enzyme were detected as different subunits in both SA1 and SPA1 genomes including, subunits alpha (*ureC*), beta (*ureB*) and gamma (*ureA*), while only the alpha subunit was found in SDG4. Furthermore, genes encoding for urease accessory proteins were observed in all genomes like, *ureD*, *ureE*, *ureF* and *ureG*. Skin infection causing bacteria have the ability to survive within several acidic environments including host skin (pH 4.1-5.8) (Proksch 2018). Therefore, these bacteria have evolved specific mechanisms to resist the acidity and survive within these niches. One of the main mechanisms to decrease the acid stress in bacteria is the production of urease enzyme. Urease enzyme catalyses the urea hydrolysis yielding ammonia (NH_3) and carbon dioxide (CO_2). Ammonium (NH_4^+) is produced as a result of ammonia ionization in water leading to pH increase (Zhou and Fey 2020). Urea is a nitrogenous waste product which is secreted into the urine in humans. It also exists in kidney tubules and other body fluids such as sweat and blood (Burne and Chen 2000). Thus, it is easily accessible for many bacteria like *S. aureus* which inhabits the skin and sweat glands. It was stated by Zhou and co-workers that urease deletion mutant in *S. aureus* revealed a remarkable decrease in bacterial load during a chronic renal kidney infection in mice (Zhou *et al.* 2019).

Pathogenic bacteria produce toxins that have the ability to manipulate the immune response components. These toxins are effectively secreted proteins which have a cytolytic activity, since they can damage the cell plasma membrane by forming pores in the membrane followed by a cell lysis such as, leukocidin and haemolysins. Several types of these membrane-damaging toxins were detected in all pathogenic genomes. Leukotoxins include leukocidin D-E (LukD-E) and Panton-Valentine leucocidin (PVL) which specially cause lysis to white blood cells (WBCs). While, haemolysins have the ability to lyse both red and white blood cells (Foster

2005). The toxin α-haemolysin is mainly produced by *S. aureus* and it exhibits a cytolytic activity towards human monocytes and platelets (Menestrina *et al.* 2001). Whereas γ-haemolysin are cytotoxic to erythrocytes (Kaneko and Kamio 2004).

It has been reported by several studies that pathogenic bacterial cells synthesize or secrete various compounds which may show an equivalent contribution to the virulence of pathogens including colonization and persistence during infections (Lowy 1998; Stehr *et al.* 2003). Despite the combined action of these numerous virulence factors, bacterial pathogenicity can be caused by the produced toxins alone without the necessity of the involvement of the bacterial infection (toxinoses). These toxins are correlated to particular diseases. For instance, toxic shock syndrome toxin-1 (TSST-1) causes toxic shock syndrome (TSS), and enterotoxins cause food poisoning (Arbuthnott *et al.* 1990). Genes encoding for TSS toxin-1 were found as two gene clusters (*tst_1* and *tst_2*) in both SA1 and SPA1, whereas SDG4 had only one gene (*tst*). Several genes encoding for different types of enterotoxins (*ent*) were observed as clusters in all genomes of the three pathogens including types A, C, D, E, G, and H. Both of TSST-1 and enterotoxins are known as pyrogenic toxin superantigens (PTSAgs). Superantigens are a special group of exotoxins that are produced by virulent *S. aureus* and *S. pyogenes* strains (Kumar 2005). The superantigenicity of these toxins is due to their ability to induce the proliferation of T-lymphocytes by binding to the conserved portions of these cells receptors and stimulate the release of cytokines in high concentrations which caused a completely excessive inflammatory response (Lina *et al.* 2004; Holtfreter and Broker 2005). It has been declared by Fisher and co-workers that the best investigated mechanisms for the pathogenicity of enterotoxins are their superantigenic and enterotoxigenic effects (Fisher *et al.* 2018). Other toxins produced by *S. aureus* can also cause diseases without the contribution of the bacteria like, PVL that causes skin diseases and necrotizing pneumonia (Gillet *et al.* 2002), the exfoliative toxins A and B (ETA and ETB) which are the cause of scalded skin syndrome (SSS), atopic dermatitis and impetigo (Lina *et al.* 1997; Capoluongo *et al.* 2001). Neither PVL nor exfoliative toxins were detected in the SA1 isolate involved in the recent study.

Lantibiotics encoding genes were found in SA1 and SPA1 genomes. Bacteriocins are classified into two groups of peptides, class I that have been subjected to post-translational modification, and class II which are unmodified peptides. Lantibiotics belong to class I bacteriocins (Cotter *et al.* 2013). A large number of Gram-positive bacterial species produce lantibiotics such as *Staphylococcus* and *Streptococcus* to attack other Gram-positive bacteria (Pag and Sahl 2002; Cotter *et al.* 2005a). Lantibiotics such as gallidermin reveal a remarkable

in vitro activity against clinical pathogens like, staphylococci including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and *Streptococcus pneumoniae* (Piper *et al.* 2009).

Genes encoding for M protein were detected in SPA1 and SDG4 sequenced genomes. M protein is an important virulence factor in streptococci that has strong determinants and stimulates type-specific antibodies in human serum to defend against infection (Terao *et al.* 2005). Depending on the variable antigenic characteristics of the major surface M protein, group A streptococci (GAS) are divided into serotypes. More than 100 types of M protein have been defined. Each Fn-binding protein is expressed in a specific group of M serotype strains (Natanson *et al.* 1995; Terao *et al.* 2001). Expression of one or more Fn-binding proteins in *S. pyogenes* strains which belong to a particular serotype, indicates the high virulence of that serotype. Serotype M1 includes the strongest pathogenic strains which possess FbaA protein and cause severe infections. However, there are also other highly virulent serotypes (Johnson *et al.* 1992; Inagaki *et al.* 2000). For instance, FbaB protein was found to be only produced by invasive infectious strains such as those of M3 and M18 serotypes. These two serotypes have been isolated from patients with aggressive diseases (Cunningham 2000; Terao 2012).

3.6 Conclusions

1- Both dairy (yogurt) and non-dairy (olives) food products used in this study contained species of lactic acid bacteria, indicating that fermented products existed in the local markets of Baghdad city were considered as rich isolation sources of probiotics.
2- Using the 16S rRNA gene sequencing technique, the major *Lactobacillus* species isolated from yogurt was identified as *Lb. delbrueckii*, while *Lb. plantarum* was the predominant recognised species in olive samples.
3- Several genes encoding for antimicrobial compounds were detected in *Lactobacillus* sequenced genomes isolated from food samples, in addition to a number of genes responsible for stress resistance. All these genes provide the food isolates with promising probiotic characteristics, indicating that food lactobacilli may represent as effective probiotic candidates.
4- Numerous genes encoding for virulence factors were recognised in the sequenced genome of each pathogen. Since the three pathogens were Gram-positive bacterial species, most of the identified genes were found in all sequenced genomes. However, a couple of genes were recognised in the single pathogenic genome but not in all genomes. Those genes were

responsible for a particular metabolic activity or the virulent capability associated with that pathogen, such as staphylocoagulase in *S. aureus* (SA1) and M protein in both *S. pyogenes* (SPA1) and *S. dysgalactiae* subsp *equisimilis* (SDG4).

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Chapter Four

Bacterial Diversity Profiling of Food Samples

4 Bacterial Diversity Profiling of Food Samples

4.1 Introduction

To investigate the bacterial biodiversity of food products, next generation sequencing (NGS) technique was carried out for food samples used in the thesis: yogurt and olives. Furthermore, to detect the difference of bacterial diversity, NGS was carried out for food samples which were cultivated in an enrichment medium and incubated under both aerobic and anaerobic conditions. Exploration of the bacterial species available in food samples was conducted to compare between both NGS and culture-dependent methods.

Taxonomic or phylogenetic classification of DNA sequences is the main step to understand the microbial community structure, as well as the relation between organisms and their environments. Most investigations of microbial populations within an individual community like soil, open ocean and human gut, have depended on a single gene: the 16S small subunit ribosomal RNA (rRNA) gene (Costello *et al.* 2009; Gilbert *et al.* 2012). In addition to the amplification of this gene, a wide range parallel sequencing techniques are progressively applied to the characterization of microbial communities resulting in a better evaluation of present biodiversity (Sogin *et al.* 2006). The 16S rRNA gene exists in all bacteria and archaea but not in eukaryotes (which have a homologous gene, the 18S rRNA gene). This gene has variable regions that assist in the taxonomic classification. It has also conserved regions which are the binding sites for PCR primers.

Application of high-throughput 16S rRNA gene sequencing to food environments has been used for exploring bacterial populations in food samples and the food production matrices (Delcenserie *et al.* 2014; Pothakos *et al.* 2014). These investigations focus on the benefit of 16S rRNA gene sequencing to explain complex bacterial communities available in food. In environmental communities, it is essential to accurately identify and classify microbes to the genus or species level. However, this level of classification is more important for pathogenic bacteria identification for human health and food safety purposes. Application of 16S rRNA gene sequencing on food samples is not designed to identify single species of specific bacterial pathogens, but it could allow simultaneous identification of any available human pathogens that were not previously defined (Ceuppens *et al.* 2017). 16S rRNA gene sequencing has been applied more broadly for intestinal microbiota analysis. The reported findings show that the

microbiome composition may be influenced by variations in the experimental procedures for nucleic acid preparation and purification, to bioinformatics analysis (Hiergeist *et al.* 2015; Hiergeist *et al.* 2016). NGS and metagenomics have been currently applied in food microbiology and their uses are increasing.

Food microbial populations differ according to the type of food. Dairy products are considered an ideal environment for the growth of microorganisms. Milk is an example of dairy products, it is extremely nutritive and has a pH which is approximately neutral (Quigley *et al.* 2013a). Raw milk microbiota is relatively complicated with a diverse microbial community affected by hygiene, season, animal health, animal species and other different factors (Quigley *et al.* 2013b). Milk supply chains includes several stages which are: production, transport, processing, packaging, distribution, retail and consumer. One of the microorganisms that can cause milk contamination on farms or during dairy processing steps is the spore-forming bacteria. These bacteria can form endospores which enable them to resist and survive within harsh ecological conditions (Logan and De Vos 2009; Postollec *et al.* 2012), such as: extreme heat or cold, food shortage, pressure, drought, biocides and ultraviolet irradiation (Moeller *et al.* 2008). Spore-forming bacteria are found in many environments, but commonly in the soil, also they naturally colonize the gastrointestinal tract of insects and warm-blooded animals (Postollec *et al.* 2012). They are Gram-positive bacteria consisting of more than 200 species belonging to the phylum Firmicutes which is divided into seven different classes including Bacilli and Clostridia (Moeller *et al.* 2008; Zhang and Lu 2015). With continuous evolution and re-classifications, these two classes have the highest dominance within Firmicutes phylum (Galperin 2013) and are the most important classes associated with the dairy industry. *Bacillus* and related species are aerobic spore-forming bacteria that have a significant influence on the food quality and safety. Furthermore, they affect the food as spoilage-causing microorganisms and they also have the potential to cause diseases (Gopal *et al.* 2015). *Bacillus* sp. is mainly found in soil which is considered as a reservoir for this bacteria (Hong *et al.* 2009). *Bacillus* sp. has extensive physiological characteristics which allow species colonization in all of the natural environments including soil, water, air, lake sediments and animal feed. Moreover, these species can colonize the extreme environments such as: hot spring, thermal acid water, sub-Antarctic soil and diseased bee larvae (Claus and Berkeley 1986). Aerobic spore-forming bacteria are the main concern for the dairy industry because of their abilities to cause significant spoilage and less for their pathogenicity. These microbes have an important impact on the quality and safety of food products through three different mechanisms: toxin production; spoilage e.g. enzyme

production and influencing the secondary dairy production such as yogurt, cheese and milk powders (De Jonghe *et al.* 2010).

In terms of agricultural products, table olives are one of the main products which is consumed fermented. The initial purpose of fermentation is to reach a preservation effect of the processed product (Sánchez Gómez *et al.* 2006). To determine the quality and sensory features of the final product, variable microbial groups are involved in olive fermentation. However, lactic acid bacteria and yeast are the most commonly associated microorganisms controlling the fermentation process (Arroyo-López *et al.* 2008; Hurtado *et al.* 2012). Understanding the diversity of lactic acid bacteria in table olive processing has constantly been performed by the development and application of new identification and typing techniques of both culture-dependent and independent procedures (Abriouel *et al.* 2011; Hurtado *et al.* 2011). Moreover, sources of lactic acid bacteria from common indigenous food products have been assessed in the context of the work carried out in this study. Several studies associated with fermented food showed that *Lb. plantarum* and *Lb. pentosus* have been recognized as the predominant species in the fermentation of table olives (De Bellis *et al.* 2010; Abriouel *et al.* 2011).

4.2 Aims

1- To characterise the bacterial community of fermented food products (yogurt and olives), specifically LAB, using NGS technique to profile 16S rRNA gene sequences.
2- To detect the variety of bacterial species community of fermented food products inoculated in two different cultural media under both incubation conditions (aerobic and anaerobic).
3- To compare the results obtained from both culture-independent method (NGS) and culture-dependent method conducted in chapter 3 for the cultivation of LAB.

4.3 Methods

4.3.1 DNA Extraction from Non-Cultured Fermented Food Products

Food samples used for the isolation of LAB were kept at 4ºC. Yogurt and olive samples were inoculated at the percentage of 1% (w/v) in two sets of tubes containing MRS and fastidious anaerobe (FA) (Lab M Ltd/Neogen) broth media. Each of the food products was inoculated twice in two tubes of each individual medium. These samples were labeled as 1M, 1F, 2M, 2F, 3M and 3F. The letter M indicates MRS medium, while letter F represents FA medium. All the

twelve tubes were incubated at 37°C for 18h, however, the first group of six tubes were incubated aerobically and labeled as 1MA, 1FA, 2MA, 2FA, 3MA and 3FA. Whereas the second six tubes were incubated under anaerobic conditions in the anaerobic cabinet (Section 2.2.1) and named as 1MAn, 1FAn, 2MAn, 2FAn, 3MAn and 3FAn. After incubation, the tubes were centrifuged at 2000 g for 20 min and the supernatants were discarded. Extraction of the genomic DNA from all the above tubes was carried out using Maxwell 16 Tissue Purification Kit (Promega) and Maxwell instrument (see chapter 2, section 2.4.1). For the amplification of 16S rRNA gene, PCR was performed for the extracted DNAs using the universal primers; forward 27F and reverse 1492R) (see chapter 2, section 2.4.2). Following that, PCR products were visualized by gel electrophoresis (see chapter 2, section 2.4.3).

4.3.2 Sequencing of 16S rRNA Gene Amplicons

The Qubit fluorometer (Invitrogen) and the Qubit dsDNA BR (Broad-Range) Assay Kit (Invitrogen) were used for the quantitation of the concentrations of the genomic DNAs (section 4.2.1). To investigate the diversity of the food bacterial community of these samples, next-generation sequencing of the 16S rRNA gene was performed for samples inoculated in both MRS and FA broth (Kindinger *et al.* 2017). The genomic DNAs of only seven samples (that revealed clear PCR products) were sequenced at the Division of Digestive Diseases, Department of Surgery and Cancer, Imperial College London by Dr Julie McDonald. For the preparation of libraries for samples, Illumina 16S Metagenomics Sequencing Library Preparation Protocol (Vo and Jedlicka 2014) was used with some modifications. To target the V1-V2 region of the 16S rRNA gene, the amplification was done using the primers listed in table 4.1. The reverse primer is 388R, while the forward primer was a formulation of four primers, which were mixed at a 4:1:1:1 ratio, it was prepared by mixing four parts of the primer 28F-YM and one part each of the three primers specific for the amplification of *Bifidobacterium* (28F-Bif), *Borrelia* (28F-Bor) and *Chloroflexus* (28F-Chl). These primers were used to characterise the bacterial community of food samples specifically LAB. Cleaning up and normalization of the index PCR reactions were done by SequalPrep Normalization Plate Kit (Life Technologies, Paisley, UK). Finally, sample libraries were quantified using the NEBNext Library Quant Kit for Illumina (New England Biolabs, Hitchin, UK). Sequencing was accomplished on an Illumina MiSeq platform (Illumina Inc., Saffron Walden, UK) using the MiSeq Reagent Kit v3 (Illumina) using paired-end 300bp chemistry.

The resulting 16S rRNA gene sequences were analysed by Dr Ann Smith using the MiSeq standard operating procedure (SOP) pipeline of the Mothur bioinformatic software package version 1.35.1 (Schloss *et al.* 2009) to analyse the samples on a single run (Kozich *et al.* 2013). SILVA bacterial database was used to perform the sequence alignment (Quast *et al.* 2012). To obtain the operational taxonomic units (OTUs) of each of the seven samples, taxonomic binning and classification were acquired at the phylum, genus and species levels using reference sequence files from both the Ribosomal Database Project (RDP) (Maidak *et al.* 2000) and SILVA (Quast *et al.* 2012) databases, using a cut off value of 97% (Appendices 3 and 4). To show the abundance percentages of the obtained OTUs, results were graphically displayed by using Phyloseq package in RStudio software version 1.2.1335 (Appendix 5) (McMurdie and Holmes 2013).

Table 4.1 Oligonucleotide primers used for NGS of 16S rRNA amplicon

Primer	Oligonucleotide Sequence (5′ – 3′)	References
338R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGCTGCCTCCCGTAGGAG	(Song *et al.* 2013)
28F-YM	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAGTTTGATYMTGGCTCAG	(Walker *et al.* 2015)
28F-Bif	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGGTTCGATTCTGGCTCAG	(Walker *et al.* 2015)
28F-Bor	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAGTTTGATCCTGGCTTAG	(Walker *et al.* 2015)
28F-Chl	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAATTTGATCTTGGTTCAG	(Walker *et al.* 2015)

4.4 Results

4.4.1 Estimation of the Bacterial Diversity of Food Samples

Genomic DNA was extracted from all food samples and the extracted DNA was quantified before sending the samples for NGS analysis. Table 4.1 show the DNA concentration of food samples inoculated in both MRS and FA medium under both incubation conditions. Concentrations of extracted DNA were low (0.01 µg/ml) in food samples: 1MAn (yogurt), 3MAn, 3FA and 3FAn (Altunsa olive). DNA concentration was slightly higher ranging between 0.02-0.16 µg/ml for other yogurt samples including: 1MA, 1FA and 1Fan. Whereas DNA could not be quantified in Zer olive samples because of either very low concentration or absence of extracted DNA in theses samples. These findings are all clear by observation of corresponding PCR product bands in agarose gel electrophoresis (Figures 4.1 and 4.2).

After amplification of 16S rRNA gene, gel electrophoresis was separately carried out for PCR products of the samples inoculated in MRS broth (Figure 4.1) and of those inoculated in FA broth (Figure 4.2). Samples of the first food product (P1: thick yogurt) and the third food product (P3: Altunsa olive) revealed clear bands in the agarose gel. These samples are 1MA, 1MAn, 3MAn, 1FA, 1FAn, 3FA and 3FAn. Whereas no bands were shown for PCR products of the second product (P2: Zer olive). There were also no bands for the Altunsa olive sample incubated aerobically in MRS broth (3MA). To explore the bacterial diversity in fermented food products, next-generation sequencing of the 16S rRNA gene was performed on the samples that show clear bands in gel electrophoresis. After using Mothur bioinformatics tool to analyse the sequences of 16S rRNA gene amplicon, SILVA and RDP databases were used to perform the taxonomic binning to acquire the operational taxonomic units (OTUs) and the bacterial diversity percentages of the seven food samples at the phylum, class, order, family, genus and species levels (Appendix 4).

Table 4.2 Concentration of extracted genomic DNA using Qubit Fluorometer and the Qubit dsDNA BR (Broad-Range) Assay Kit

Food Sample	DNA Concentration (µg/ml)
1MA	0.02
1MAn	0.01
3MAn	0.01
1FA	0.16
1FAn	0.11
3FA	0.01
3FAn	0.01

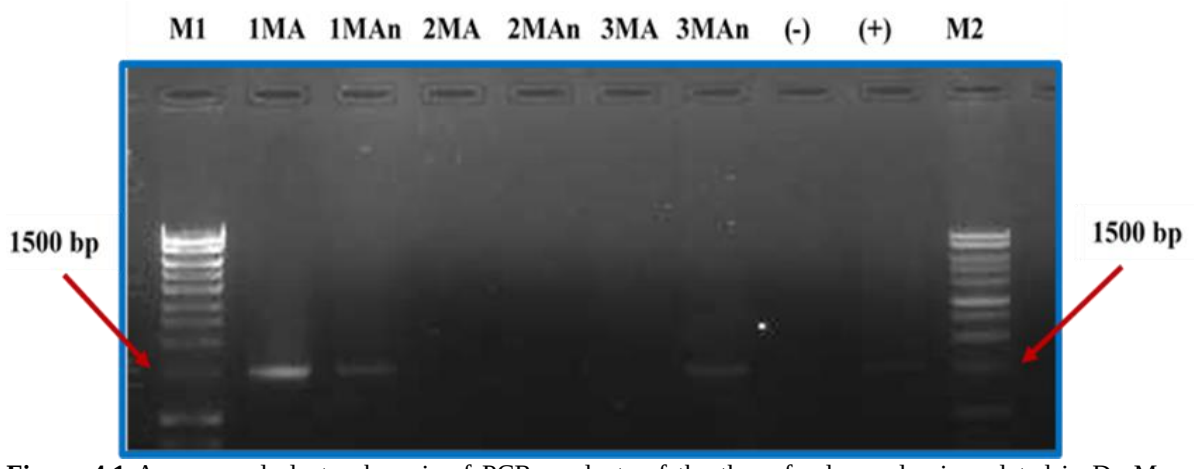


Figure 4.1 Agarose gel electrophoresis of PCR products of the three food samples inoculated in De Man Rogosa Sharpe (MRS) broth under aerobic (A) and anaerobic (An) conditions; Lane M1: Marker1 (hyper ladder1kb), Lanes 1MA-3MAn: food samples, Lane M2: Marker 2 (ladder 1kb), (-): -ve control, (+): +ve control

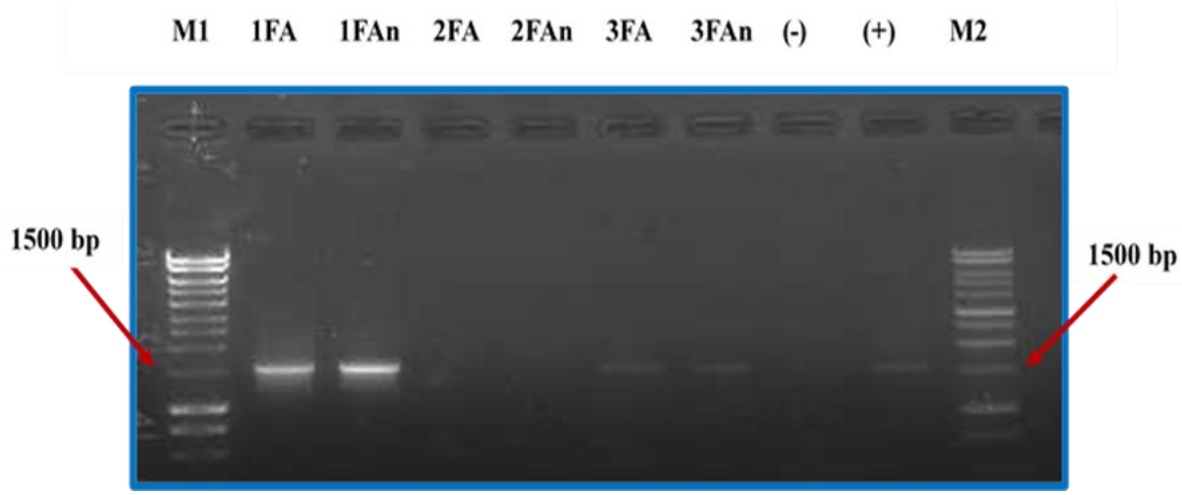


Figure 4.2 Agarose gel electrophoresis of PCR products of the three food samples inoculated in fastidious anaerobic (FA) broth under aerobic (A) and anaerobic (An) conditions; Lane M1: Marker1 (hyper ladder1kb), Lanes 1FA-3FAn: food samples, Lane M2: Marker 2 (ladder 1kb), (-): -ve control, (+): +ve control

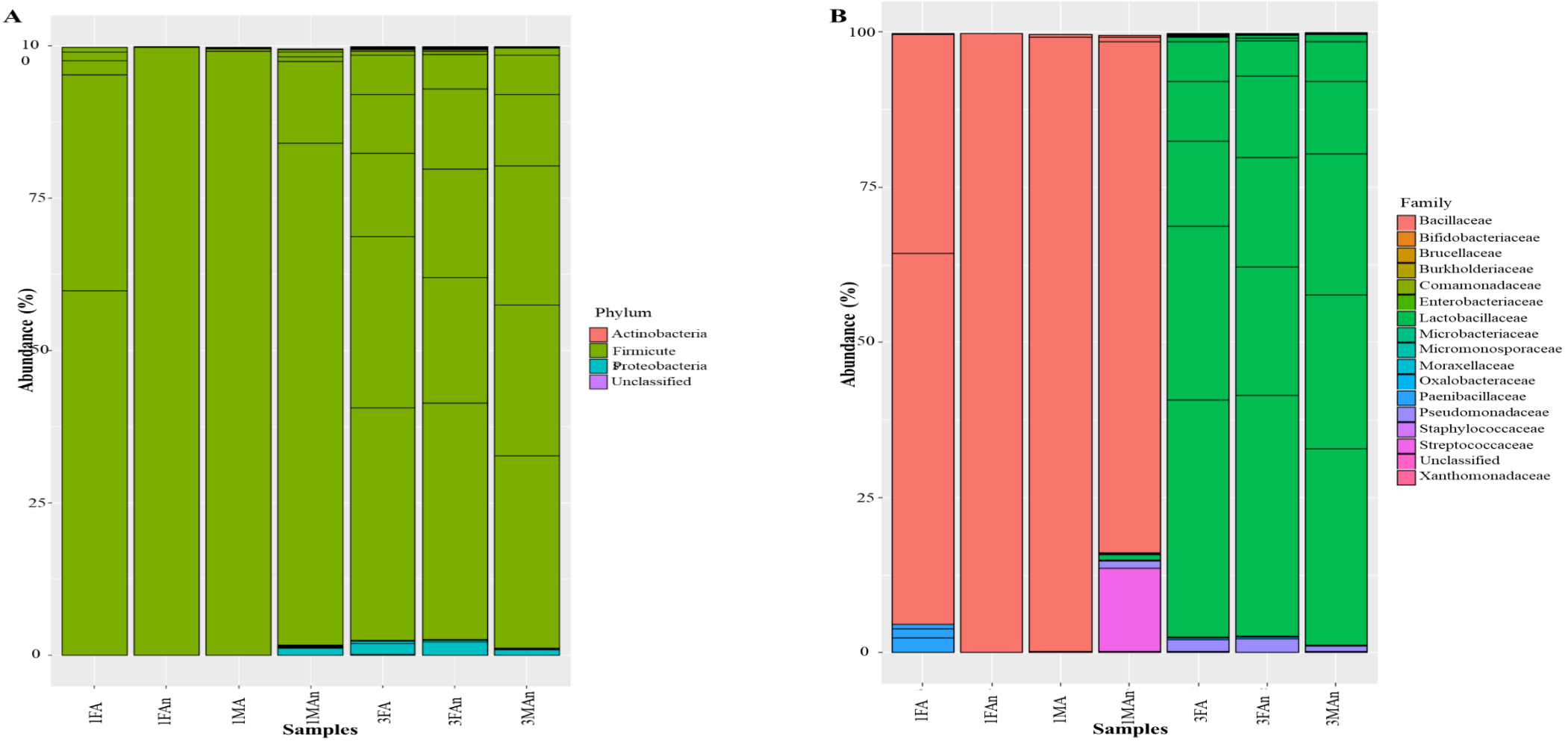
Yogurt samples (1FA, 1Fan, 1MA and 1MAn), cultivated in both media and incubated, either aerobically or anaerobically, revealed high abundance of the phylum Firmicutes (Figure 4.3A) and family Bacillaceae (Figure 4.3B). *Bacillus* was the predominant genus in all samples with the appearance of different species of this genus (Figure 4.3C). Figure 4.3D displays the species abundance within yogurt samples. Sample 1FA revealed *B. licheniformis* and *B. amyloliquefaciens* with abundance percentages of (59.8%) and (35%), respectively. The genus *Paenibacillus* that refers to Paenibacillaceae family was also present in this sample, but only in small percentages (0.7% - 2%) of three species: *P. relictisesami*, *P. cookii* and *P. timonensis*. Among all yogurt samples, *Bacillus* showed the maximum predominance as *B. cereus* in 1FAn (99.8%) and as *Bacillus* sp. in 1MA (99%). While the main prevalent species in sample 1MAn was *Bacillus* sp. (82%) followed by *Streptococcus thermophilus* (13%) which belongs to Streptococcaceae family. This yogurt sample also contained low abundance of the phylum Proteobacteria, specifically *Pseudomonas aeruginosa* (1.1%) which belongs to Pseudomonadaceae family. Under the same phylum, there was a very low prevalence of Enterobacteriaceae family, in particular *Shigella dysenteriae* was indicted in samples 1FA and 1MAn with percentages of 0.004% and 0.02%, respectively. While only sample 1MAn revealed low abundance (0.02%) of *Staphylococcus aureus* within the family Staphylococcaceae. Regarding the presence of lactic acid bacteria in yogurt samples, low prevalence of four *Lactobacillus* species was observed in some of these samples. *Lb. rapi*, *Lb. parafarraginis* and *Lb. acidipiscis* all revealed the same abundance (0.004%). *Lb. delbrueckii* was isolated from

yogurt (inoculated in MRS medium) by culture-dependent procedures. However, NGS method showed low prevalence of this species as *Lb. delbrueckii* subsp. *indicus*. Yogurt samples of the same medium incubated both aerobically and anaerobically showed abundance percentages of (0.01%) and (0.8%), respectively.

In terms of the olive-based food samples (3FA, 3FAn and 3MAn), the Firmicutes phylum and Lactobacillaceae family were the most prevalent (Figures 4.3A and 4.3B). Particularly, the genus *Lactobacillus* which revealed high abundance in all of these food samples (Figure 4.3C). Regardless of the medium type and incubation conditions used, a total of seven defined *Lactobacillus* species were detected with different abundance ranges among the samples, as well as other *Lactobacillus* species (Figure 4.3D). These species were: *Lb. rapi* (31.6% - 38.8%), *Lb. buchneri* (11.7% - 28%), *Lb. parafarraginis* (13% - 22.7%), *Lb. pentosus* (5.7% - 24.8%), *Lb. acidipiscis* (6.5% - 17.7%), *Lb. vaccinostercus* (0.1% - 0.3%), *Lb. namurensis* (0.004% - 0.1%) and *Lactobacillus* sp. (0.01% - 0.1%). *Lb. plantarum* was previously isolated from olives by culture-dependent methods (Chapter 3). Nevertheless, the analysis of the NGS data showed no abundance of this species within the genomic DNA extracted from the olive-based samples. Under the same family Lactobacillaceae, *Pediococcus* was found at low abundance as *P. ethanolidurans* (0.5% - 1.2%) and *P. parvulus* (0.01%-0.03%). Different *Bacillus* species also showed very low abundance (0.004% - 0.2%) in olive-based samples. *S. aureus* was also found at low levels (0.03% - 0.1%). The Proteobacteria phylum was also present in these samples, especially *P. aeruginosa* with a prevalence of (0.9% - 2%) and *S. dysenteriae* with an abundance of (0.004% - 0.01%).

Regarding different *Lactobacillus* species found in both yogurt and olives, Figure 4.4 displays the abundance percentages of all these species using two 16S rRNA gene databases SILVA and RDP. These species were divided into two groups depending on their abundance. The first group was lactobacilli with abundance percentages ranging between 0% - 40% of the total bacterial sequences (Figures 4.4A). The second group was lactobacilli that are abundant in low percentages (0% - 1%) (Figures 4.4B). *Lb. rapi* had the highest abundance (38.8%) in the Altunsa olive food when inoculated in FA broth and incubated under anaerobic conditions (3FAn). These percentages were the same by means of both SILVA and RDP databases. MRS medium is generally used for the isolation of LAB from most fermented food products and it is designated as a medium for LAB cultivation. Despite this fact, FA medium also showed high variability as a growth medium for LAB especially olive samples under both aerobic and anaerobic conditions. Anaerobic conditions enhanced the growth of *Lb. delbrueckii* subsp.

indicus more than the aerobic conditions, in particular when the yogurt sample was inoculated in MRS broth. This was observed by the increased percentage of species prevalence in MRS incubated under anaerobic conditions.



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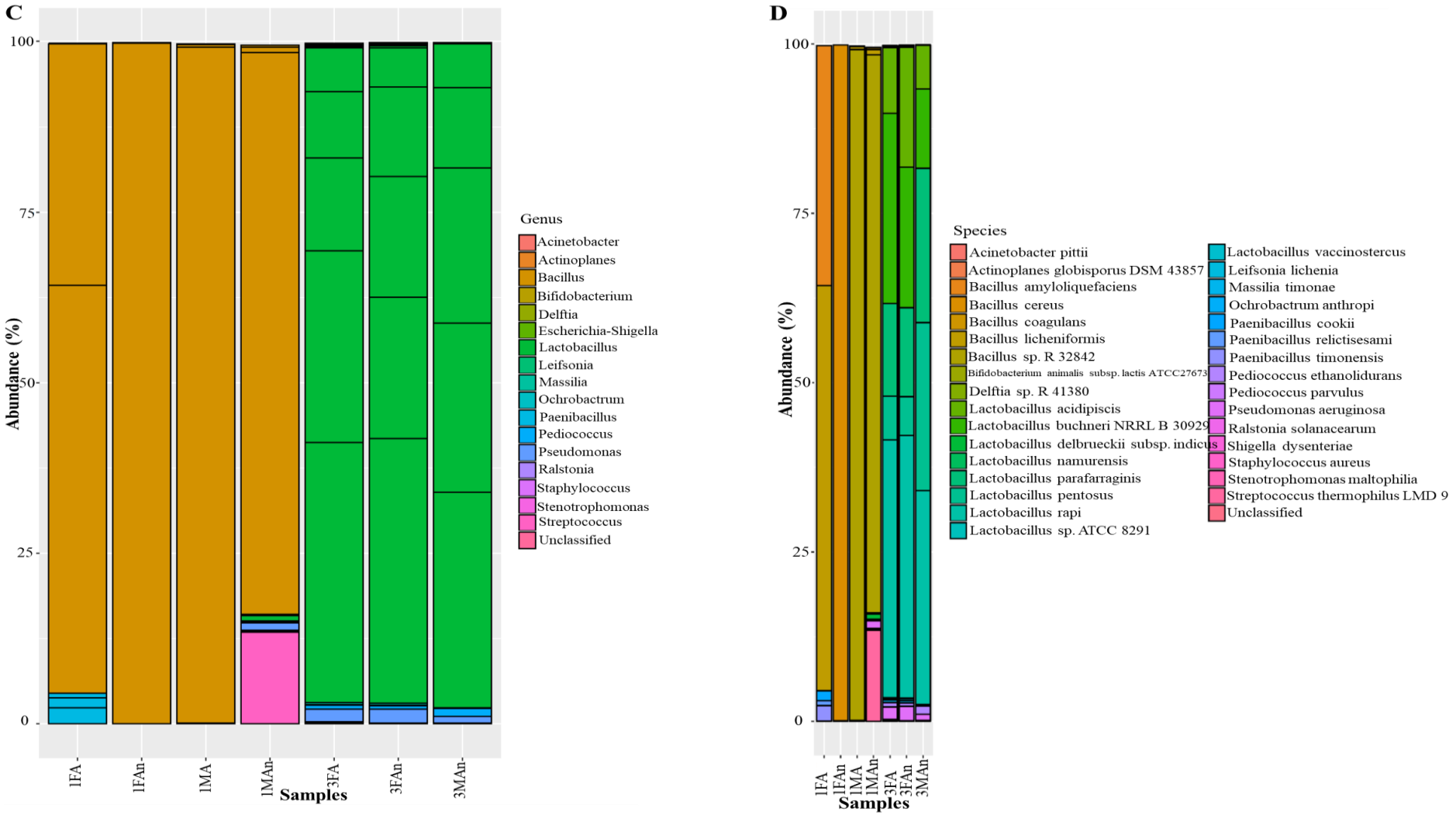

Figure 4.3 (A) Phyla taxonomic classification results obtained by NGS of 16S rRNA gene for food samples using Phyloseq package in R statistical software. (B) Families taxonomic classification results obtained by NGS of 16S rRNA gene for food samples using Phyloseq package in R statistical software. (C) Genera taxonomic classification results obtained by NGS of 16S rRNA gene for food samples using Phyloseq package in R statistical software. (D) Species taxonomic classification results obtained by NGS of 16S rRNA gene for food samples using Phyloseq package in R statistical software. The abundance values of each OUT are stacked in order from greatest to least, separate by thin horizontal lines.

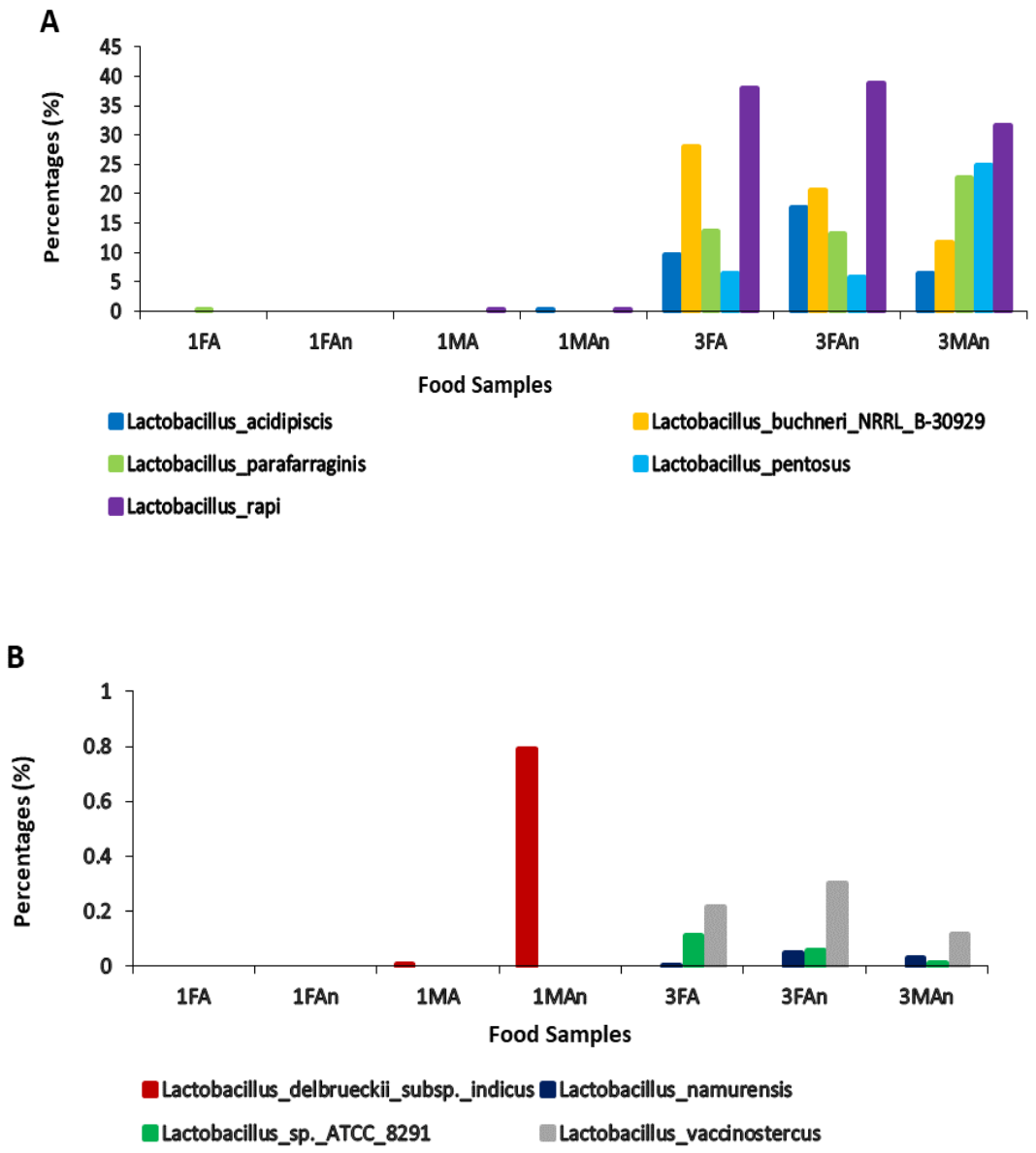

Figure 4.4 Distribution of Lactobacilli species in food products samples (1: yogurt, 3: Altunsa olive) grown in two different broth media (M: MRS and F: FA) under two incubation conditions (A: Aerobic and An: Anaerobic) and identified by sequencing of 16S rRNA region using both SILVA and RDP. (A) Lactobacilli group with abundance percentages ranging between 0% - 40%. (B) Lactobacilli group that are abundant in low percentages (0% - 1%)

4.5 Discussion

Sequence analysis of the 16S rRNA gene is a reliable tool used for the genomic characterization of bacterial diversity. Furthermore, to identify an organism by 16S rRNA gene sequencing, there is no necessity for the cultivation of this organism (Dethlefsen *et al.* 2007; Turnbaugh *et al.* 2007). Next-generation sequencing (NGS) is a promising method for microbial characterization in complex populations depending on functions (i.e. functional genes) and/or taxonomy (i.e. taxonomic diversity and relative abundance of 16S rRNA gene sequences) (Guo *et al.* 2013).

NGS analysis data showed that *Bacillus* was the most predominant bacteria in all yogurt samples tested for 16S RNA gene sequence. Several species of *Bacillus* were detected among these samples such as: *B. cereus*, *B. licheniformis*, *B. amyloliquefaciens* and *Bacillus* sp. Most members of this genus are non-pathogenic, while *B. anthracis* and *B. cereus* are considered as pathogens. A deep analysis into the *Bacillus* genus has been performed by De Jonghe and colleagues who declared that species other than *B. cereus* are not found in food poisoning cases (De Jonghe *et al.* 2010). However, the production and functionality of heat-labile toxins by *B. licheniformis* (Lindsay *et al.* 2000) and *B. amyloliquefaciens* (Phelps and McKillip 2002) have been confirmed by cellular investigations of these cases. It was indicated by (Tolle 1980) that healthy udder cells contain sterile milk. Nevertheless, milk can be contaminated by numerous sources like: the external surface of animals; or during milking, transfer, storage or processing. Determination of raw milk quality is done by assessing a combination of numerous factors such as: milk composition, udder health and sanitation (O'Brien *et al.* 2009). One of the microbiological tests performed to monitor the quality and safety of milk and other dairy products is quantifying of thermoduric bacteria and particular pathogens, like *B. cereus* (Law *et al.* 2015). Although this bacterium is a soil-inhabitant pathogen, it is frequently found in raw milk and other dairy products. Numerous pathogenic compounds are produced by different strains of this species like: enterotoxins, phospholipases and haemolysins (Turnbull *et al.* 2002). Since *B. cereus* is a spore-former bacterium, it can resist industrial pasteurization process and has an impact on the shelf-life of pasteurized milk and cream (Griffiths 1992). It was mentioned by Ghelardi and co-workers that *B. cereus* has been a recognised causative agent of food poisoning for more than 40 years (Ghelardi *et al.* 2002) and it is the only pathogen that causes food poisoning among all *Bacillus* species, while numerous other species have been identified as causatives of bacterial spoilage of milk and milk products. *B. licheniformis* is also one of the

most predominant species found in raw milk and along the dairy processing steps (Scheldeman *et al.* 2006).

Though this species is not considered as a human pathogen, its spores are able to cause milk and dairy products spoilage, affecting milk functional features (Dhakal *et al.* 2014). It has been established by previous food studies that *B. licheniformis* strains are highly prevalent in the dairy industry. This predominance is related to contamination caused either by external factors like silage and soil or other sources within dairy processing factories (Scheldeman *et al.* 2005; Dhakal *et al.* 2014). The significant dominance of this species in milk samples is possibly associated with its wide spread existence in the environment and across dairy farms (Vaerewijck *et al.* 2001; Scheldeman *et al.* 2005). As *Bacillus* sp. are ubiquitous bacteria in nature, it can be difficult to define the actual contamination source by these bacteria in dairy farms. However, contamination may occur through the milk chain and the most known sources involve soil, silage, feed, faeces and rinse water from milking equipment (Magnusson *et al.* 2007). The whole milk processing continuum would be influenced when these spoilage species contaminate the raw milk. This is due to the ability of some *Bacillus* sp. to form spores which have high hydrophobic characteristics allowing them to adhere to the materials used in food processing e.g., stainless steel, forming multicellular structures called biofilms (Husmark and Rönner 1992). The genus *Paenibacillus* was also found in low percentages in one of the yogurt samples. This genus was proposed by Ash and colleagues after performing a comprehensive study into the 16S rRNA gene sequences of 51 species of the *Bacillus* genus (Ash *et al.* 1991). Strains of this bacterium can normally inhabit water, soil, plants rhizosphere, diseased insect larvae and food products (Ahn *et al.* 2014). Heyndrickx and Scheldeman (2002) declared that low numbers of *Paenibacillus* spores are possibly present in both raw and pasteurized milk. *Paenibacillus* strains can contaminate the raw milk through several sources within the dairy farm especially cattle feed and silage (Te Giffel *et al.* 2002). *Bacillus* sp. and *Paenibacillus* sp. are both able to tolerate and survive the high temperature short time (HTST) pasteurization procedures affecting the shelf-life of HTST pasteurized fluid milk (Huck *et al.* 2007; Huck *et al.* 2008).

Differences between molecular and culture techniques are frequently detected in food microbiology because of several reasons associated with the microbe and the food environment (Ceuppens *et al.* 2014). Microbiota characterization by culture-dependent methods might not represent the complete microbial diversity of complicated matrices. Microbial populations which reveal higher abundance can inhibit the growth of the less abundant species. Furthermore, bacteria may be underestimated by culturing methods when the bacterial growth is lower than

the level required for detection or the culture medium is sub-optimal. Underestimation of bacteria occurs because the bacteria are in a stressed state or due to the existence of inhibitory compounds producing microbes (Cocolin *et al.* 2007; Jasson *et al.* 2009). Therefore, achievement of the progressive identification and detection of microorganisms in complex food environments can be conducted by culture-independent methods based on molecular biology methods, such as metagenomic analysis using NGS (Cocolin *et al.* 2007). Although Ceuppens and co-workers mentioned several advantages of metagenomics, such as avoidance of culturing techniques, this analysis could not be detective for enteric bacteria in food samples as these pathogens are only available in low abundance (Ceuppens *et al.* 2017). Results reported in the previous research were similar to what was obtained from analysis of the present study when low prevalence of Enterobacteriaceae, especially *Shigella dysenteriae* was detected in a couple of yogurt and olive samples. Pathogenic bacteria of the Enterobacteriaceae family i.e. *Salmonella*, *Yersinia* and pathogenic *Escherichia coli* play an important role in foodborne disease particularly for food of both animal and non-animal origin (European_Food_Safety_Authority_Panel 2013). The accurate identification of these bacteria using the V1, V2, V3 and V6 regions can be less well identified (Chakravorty *et al.* 2007; Guo *et al.* 2013). In contrast, nucleic acids from dead cells may be detected by molecular techniques which resulted in overestimation of the incidence of foodborne pathogens (Jasson *et al.* 2009).

In a study conducted for the detection of Shiga toxin-producing *E. coli* on freshly bagged spinach, Leonard and colleagues stated that an enrichment procedure is necessary before performing NGS analysis. They mentioned that the importance of both techniques is to enable targeted pathogens for growing in higher numbers and to detect the toxin production (Leonard *et al.* 2015). NGS of 16S rRNA gene amplicons was carried out by Ceuppens and co-workers to identify the bacteria present on fresh basil leaves. This investigation revealed few reads allocated to *Salmonella*, however the accuracy of these results was doubted since the taxonomy based on short (500 bp) fragments of 16S rRNA region V1-V3 is not efficient to distinguish *Salmonella* from other closely related enterobacterial species (Ceuppens *et al.* 2017). Adeola and colleagues declared that it is difficult to understand the relation and phylogeny of the genera within the order Enterobacteriales depending on 16S rRNA gene or other single-gene or even multi-gene approaches (Adeolu *et al.* 2016). Few OTUs with very low numbers of reads could significantly increase the possibility of artefacts and consequently incorrect detection. Therefore, to exclude the results originated from artefacts, determination of the threshold value is important, e.g. reads consisting ≤ 1% of the total (Ceuppens *et al.* 2017). To remove artefacts in this study, all OTUs

less than 10 reads were removed and labelled as OTU X. Human pathogens are available in low percentages in food samples which usually include natural bacterial populations. Thus, to increase the possibility of detecting low prevalent pathogenic bacteria in food, application of deep sequencing procedures with very high coverage is important. Nevertheless, the analysis cost would significantly increase by using deep sequencing.

Several pathogenic bacteria were found in yogurt and olive samples used in this research such as: *S. aureus*, *P. aeruginosa* and *S. dysenteriae*. NGS 16S amplicon sequencing is not a precise technique to detect one single particular target species in food sample, however OTU taxonomic classification outcomes designated to specific pathogenic bacterial species might be revealed. Therefore, this result is usually included within the non-targeted 16S rRNA gene sequencing analysis of the microbiome and it is important to be reported as a pathogen detection finding of concern. Further detectable techniques are required to confirm positive results for pathogens acquired by NGS 16S gene sequencing. These include either typical culture-based methods or targeted PCR detection of specific (virulence) genes within the species to achieve the acceptable confidence that is needed for results with significant public health effects (Ceuppens *et al.* 2017). Chapela and colleagues mentioned that screening and control of foodborne pathogens are usually conducted by culture-dependent procedures and biochemical reactions identification (Chapela *et al.* 2015).

Results obtained from olive samples showed high abundance of different *Lactobacillus* species. The product used in this work was black olives. These findings agreed with what was concluded by Agapi and co-workers who performed an investigation to compare between the biodiversity of LAB in black and green olives. They found a wide diversity of these bacteria in black olive-based food, more than in green olives (Doulgeraki *et al.* 2013). The high diversity in black olives is because of its processing procedure which does not include a lye treatment step as in the case of other olives types. Lye is an alternative name of sodium hydroxide (NaOH) which is used to cure many types of food including olive to make it less bitter. This finding is also similar to a recent published report investigating the role of lactic acid bacteria in table olive fermentation (Hurtado *et al.* 2012).

Even though MRS is a selective medium for lactobacilli, FA medium revealed good growth for these bacteria and other bacterial species. FA medium is used for the growth of fastidious bacteria and it is considered as an enrichment medium since it is rich in nutrients such as Vitamin K, L-cysteine and peptone mixture which are all important growth factors for many

bacteria especially anaerobes (Gould and Duerden 1983; Ganguli *et al.* 1984). NGS based on 16S rRNA gene amplicon sequencing is increasingly applied for revealing the microbial community dynamics. However, the techniques depending on 16S rRNA gene are known to be restricted by the short read length obtained, sequencing errors (Quince *et al.* 2009; Quince *et al.* 2011) and difficulties in measuring operational taxonomic units (OTUs) (Huse *et al.* 2010). In addition, evaluation of the diversity using single marker gene is challenging given the difficulty in identifying bacterial species (McDonald *et al.* 2005; Konstantinidis *et al.* 2006), as well as the limited determination of the 16S rRNA gene among closely related species.

4.6 Conclusions

1- DNA was easily extracted from two food products: thick yogurt and Altunsa olive as their PCR products revealed clear bands in gel electrophoresis. While the DNA from Zer olive could not be extracted since the flesh (inner layer) of the olive fruit was hard and not soft enough to be homogenised within the broth medium used.

2- NGS analysis of all yogurt samples showed that *Bacillus* (phylum: Firmicutes, family: Bacillaceae) was the prevalent genus, regardless of the medium and incubation conditions used. This genus appeared in several different species.

3- NGS analysis of all yogurt samples showed low abundance of lactic acid bacteria, with an appearance of four different *Lactobacillus* species in small percentages within a couple of these samples.

4- Despite the isolation of *Lb. delbrueckii* from yogurt by culture-dependent procedure (using MRS medium), low abundance of this species was found using culture-independent method as *Lb. delbrueckii* subsp. *indicus.*

5- NGS analysis of all olive samples showed that *Lactobacillus* (phylum: Firmicutes, family: Lactobacillaceae) was the most prevalent genus, regardless of the medium and incubation conditions used. This genus appeared as seven defined *Lactobacillus* species with different prevalence ranges.

6- Despite the isolation of *Lb. plantarum* from olives by culture-dependent procedure (using MRS medium), no abundance of this species was detected by culture-independent method.

7- *Lb. rapi* was the most abundant species in the Altunsa olive food when it was inoculated in FA broth and incubated under anaerobic conditions.

8- Both SILVA and RDP databases revealed similar percentages of bacterial species within both yogurt and olive samples.

9- As a growth medium, FA medium presented a high diversity for the cultivation of LAB, especially for olive samples under both incubation conditions.

10- There was an obvious enhancement for the *Lb. delbrueckii* subsp. *indicus* growth in MRS broth incubated under anaerobic conditions rather than that incubated aerobically.

4.7 References

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Chapter Five

Determination of the Antagonistic Activity of Lactobacilli Against the Pathogens (*In vitro*)

5 Determination of the Antagonistic Activity of *Lactobacilli* Against the Pathogens (*In Vitro*)

5.1 Introduction

5.1.1 Probiotics and Postbiotics

To examine the antibacterial activity of *Lactobacillus* species, including both food isolates and type strains, overlay assay was carried out for thirteen different species. The growth inhibition of skin pathogens by *Lactobacillus* species was monitored during three days of incubation under both aerobic and anaerobic conditions. After determination of the incubation period which revealed the highest antagonistic activity, all *Lactobacillus* cultures (incubated anaerobically for 72h) were tested using the same ovelay method to assess the isolate which showed the maximum potency to inhibit the growth of each of the three pathogens.

Probiotic bacteria that have been defined by Havenaar and Huis In't Veld (1992) as viable bacterial cultures which show their beneficial effects by the improvement of indigenous microbiota features of the host when consumed by both human and animal. Probiotics usually occupy the the gut and promote the host health from this environment (Gibson and Roberfroid 1995; Hamasalim 2016). They are prepared as active live cultures that contain bacterial isolates from natural environments, and the genera include the lactobacilli, lactococci or bifidobacteria (Bongaerts and Severijnen 2016).

Recent information indicates that bacterial products may have similar influences on barrier function and signalling pathways as live organisms, in the absence these organisms. Bacterial products are described as postbiotics, these are non-viable metabolic by-products or that produced by probiotic microorganisms e.g., bacteria which possess biological activity in the host (Patel and Denning 2013). Postbiotics include bacterial by-products produced from metabolic activities, such as hydrogen peroxide, organic acids, bacteriocins, diacetyl, acetaldehydes and ethanol. However, it is indicated that important bacterial structures can also be maintained by certain heat-killed probiotics and these structures may utilise biological activities within the host (Islam 2016). These metabolic products can be used as alternatives to antibiotics, since they have comprehensive inhibitory features towards pathogenic microbes (Ooi

et al. 2015). Postbiotics are biproducts resulted from metabolic actions of probiotic bacteria or they are non-viable bacterial products. Because of that, they are non-pathogenic and can resist the hydrolysis caused by mammalian enzymes (Giorgetti *et al.* 2015). Identification of similar properties has been indicated in numerous probiotic species of *Lactobacillus*, *Bacteroides fragilis*, *Escherichia coli*, *Bifidobacterium lactis*, *Bifidobacterium breve* and *Bifidobacterium infantis* (Cicenia *et al.* 2014).

Despite the importance of antimicrobial agents as treatments for infectious diseases, classic antibiotics can disturb the complicated composition of the gut microbiota. Unlike antibiotics, consumption of probiotics repressed the alteration in the microbial population of microbiota. Because of this characteristic, anti-pathogenic potency is considered as one of the most valuable properties of probiotics (Tejero-Sariñena *et al.* 2013). Most of the basic clinical and nutritional supplementation can be fulfilled by probiotics, since these bacteria have significant functional features. Furthermore, they have displayed positive results as clinical treatments against many disorders and diseases, like irritable bowel syndrome (IBS), diarrhoea associated with rotavirus and food allergies (Kerry *et al.* 2018).

5.1.2 Anti-pathogenic Activity of Probiotics

Extensive research has been conducted on the anti-pathogenic effect of probiotics or probiotic mixture. Tejero-Sariñena and colleagues examined the impact of probiotics on the viability of *Salmonella enterica*, *Serovar typhimurium* and *Clostridioides difficile* (aka *Clostridium difficile*). This research group assumed that short-chain fatty acids (SCFAs) produced by probiotics can inhibit growth of pathogens (Tejero-Sariñena *et al.* 2013). These SCFAs, such as propionic, acetic, lactic and butyric acids, contribute in the maintenance of a suitable pH in the colonic lumen. This pH is essential for the expression of several bacterial enzymes and metabolism of foreign compounds and carcinogens in the gut (Kareem *et al.* 2014). It was also recommended by Islam (2016) that several probiotics have the ability to produce a wide variety of anti-pathogenic compounds, like hydrogen peroxide (H_2O_2), diacetyl, organic acids, acetaldehydes, ethanol, bacteriocins and peptides. Two of these compounds including bacteriocins and peptides have a particular mechanism that leads to the death of the target cell. They generally participate by increasing the permeability of target cell membranes, which results in the depolarization of the membrane and finally cell death (Simova *et al.* 2009). Ammor and coworkers mentioned that sulfhydryl groups existed in the composition of bacterial enzymes, can be oxidized by the effect of H_2O_2 which produced by probiotics. This oxidation leads to

denaturation of several enzymes and membrane lipid peroxidation, followed by a consequent increase in the permeability of the pathogen's membrane, resulting in the cell death (Ammor *et al.* 2006). Organic acids such as acetic and lactic acids may also have an antipathogenic potency by decreasing the pH (Kareem *et al.* 2014). Production of bioactive compounds has a direct effect on the pathogens. Furthermore, probiotics can show their effect by stimulation of the host anti-pathogenic defence pathways, like activating the production of cationic anti-microbial peptides called defensins which produced in a number of cell types of the small intestine and intestinal epithelial cells (Figueroa-González *et al.* 2011). In addition to the above, probiotics can exert their anti-pathogenic activity by the competition for existing nutrients, as well as for pathogen receptor and binding or adherent sites (Sikorska and Smoragiewicz 2013; Kerry *et al.* 2018).

5.2 Aims

1- To investigate the inhibitory activity of *Lactobacillus* species (food isolates and type strains) against the growth of skin pathogens using the overlay assay.
2- To assess the experimental factors affecting the inhibitory activity such as, the pH of *Lactobacillus* species cultures, incubation conditions of antagonism cultures and the source of *Lactobacillus* species.
3- To explore the bioactive compounds responsible for the antibacterial action showed by *Lactobacillus* species used in the study.

5.3 Methods

5.3.1 Overlay Assay

5.3.1.1 Antagonistic Activity Determination by Overlay Assay

Antagonistic activity was assessed by performing the overlay method according to the procedure of Maia *et al.* (2001) with minor modifications. For an individual lactic acid bacterial and pathogenic isolate, three to five single colonies were selected from the agar plates. Each LAB isolate was grown in 5 ml MRS broth and incubated at 37°C for 18 h under aerobic conditions. Three µl of each bacterial LAB isolate culture was spotted onto the centre of MRS agar medium and the plates were incubated anaerobically at 37°C for 24, 48, and 72 h. Before the day of experiment, the pathogenic bacteria (*S. aureus*, *S. pyogenes* and *S. dysgalactiae* subsp.

equisimilis) were suspended in 5 ml TSB and incubated aerobically at 37°C for 18 h. After each day of the three incubation days, the same steps were done for the plates spotted with LAB. They were exposed to chloroform vapour for 5 mins to kill the growing bacteria, and allowed to air for 10 mins within a laminar flow cabinet to remove the excess chloroform. Each LAB agar plate was overlaid with approximately seven ml of cooled molten soft Muller-Hinton agar (MHA) 0.7% w/v (Oxoid) seeded with 200 µl of the indicator bacteria. It was left for a couple of minutes allowing the agar to harden. To compare the inhibition activity in two different incubation conditions, a group of plates was incubated aerobically, while the other group of the same plates was incubated anaerobically at 37°C for 24 h. The sensitivity of the pathogenic bacteria was estimated by measuring the diameters of inhibition zones around the spot (Karska-Wysocki *et al.* 2010). This assay was accomplished once against the three pathogens for thirteen LAB isolates after 24 h, 48 h and 72 h of incubating the spotted bacterial cultures anaerobically. To confirm the findings and to determine the species which have the highest antibacterial activity against the tested pathogens, overlay assay was performed in triplicate for all of the lactobacilli (ten food isolates and ten type strains) after 72h incubation time, the activity was tested under both aerobic and anaerobic incubation conditions. All the obtained data were plotted and images were captured to compare between the two incubation conditions.

5.3.1.2 Antibacterial Activity of Neutralised *Lactobacillus* Cultures

As a result of acid production by *Lactobacillus* species, bacterial cultures are considered as acidic environments. Three *Lactobacillus* species were randomly selected and spotted on buffered MRS agar. This experiment was performed to neutralise the acidic effect generated by *Lactobacillus* cultures and to estimate the active compound which is responsible for some of the antibacterial activity. These isolates were: *Lb. plantarum* (Lb25) (food isolate), *Lb. casei* imunitass (Lb2) and *Lb. plantarum* LMG 6907 (Lb29) (type strains). Buffered MRS medium was prepared by using distilled water with two different molarity concentrations 0.1M and 0.01M of phosphate buffered solution (Sigma-Aldrich), separately. After incubation of the plates at 37°C for 72h, the same protocol of overlay method (Section 5.2.1.1) was repeated. This experiment was carried out once in two groups of plates. The first set was incubated aerobically, while the incubation of the second set was under anaerobic conditions. Diameters of inhibition zones were measured. Antibacterial activity of the three species cultivated on buffered medium was compared to that activity obtained by these species after their cultivation on unbuffered medium for 72h in Section 5.2.1.1 (this experiment was conducted in triplicate).

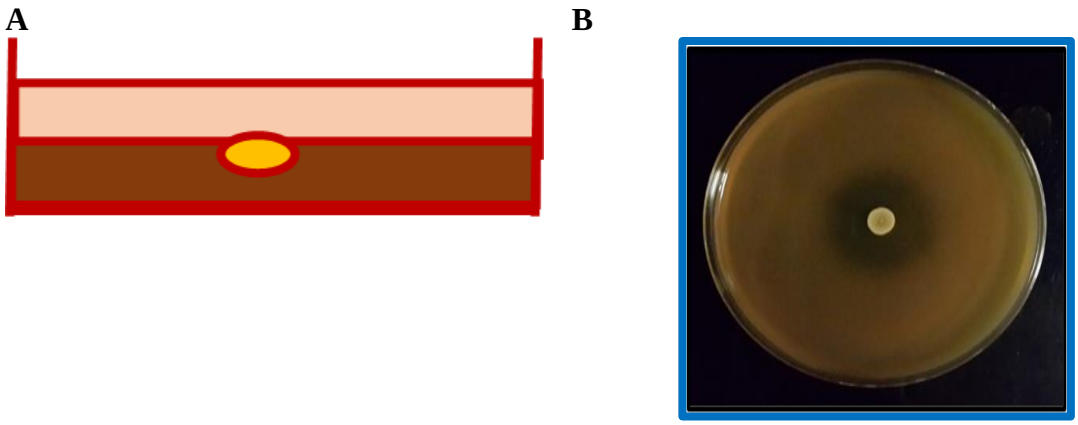


Figure 5.1 Overlay assay used for the evaluation if antibacterial activity produced by *Lactobacillus* species against pathogenic bacteria. A: a diagram of the overlay assay. B: a zone of growth inhibition resulted from the existence of an antibacterial activity of *Lactobacillus* against the pathogen

5.3.1.3 Statistical Analysis of Inhibition Zone Diameters

To compare between the inhibitory activity of *Lactobacillus* species (including food isolates and type strains) under both aerobic and anaerobic incubation conditions, statistical analysis has been carried out for diameters of inhibition zones using Two-tailed unpaired t-test in GraphPad Prism ver. 8 (GraphPad Software, Inc.). Significant differences between the two incubation conditions were detected and reported.

5.4 Results

5.4.1 Determination of the Antagonistic Activity

5.4.1.1 Determination of the Antagonistic Activity by Overlay Assay

Detection of the antagonistic activity of *Lactobacillus* species against the indicator isolates was carried out using an overlay method. Appendices 6, 7 and 8 indicate the zones of growth inhibition of *S. aureus* (SA1), *S. pyogenes* (SPA1) and *S. dysgalactiae* subsp. *equisimilis* (SDG4), respectively. This inhibition was produced by thirteen *Lactobacillus* species under two different incubation conditions; aerobic and anaerobic. Plates spotted with each isolate were incubated for 24h, 48h and 72h. Based on the findings, all tested pathogens were sensitive to the antibacterial effect of lactobacilli as they show various diameters of inhibition zones. Regardless of the incubation condition used in this assay, it has been observed that the antibacterial activity

of all lactobacilli increased through the days of incubation resulting in a maximum antagonism after 72h (Figure 5.2). Figures 5.3 and 5.4 show the increased antagonistic activity of two food isolates *Lb. delbrueckii* (Lb18) and *Lb. plantarum* (Lb23) (isolated from yogurt and olives, respectively) against two different pathogenic species (SDG4 and SA1, respectively) under aerobic condition. Whereas figure 5.5 displays the increased growth inhibition of SA1 as a result of the antibacterial potency produced by the type strain *Lb. casei* LMG 6904 (Lb27) under aerobic incubation condition.

Most *Lactobacillus* species showed high antibacterial activity against all the tested pathogens when the plates were incubated anaerobically. Furthermore, the antibacterial potential under anaerobic incubation conditions was higher than that obtained after incubating the plates aerobically. Mean and standard deviations of the inhibition zone diameters produced by all *Lactobacillus* species including food isolates and type strains were calculated and presented in Appendices 9 and 10. Figure 5.6 depicts the inhibitory activity of food isolated bacterial cultures (incubated for 72h) against each of the three pathogens under both aerobic and anaerobic conditions (5.6A, 5.6B and 5.6C for SA1, SPA1 and SDG4, respectively). In general, statistical analysis conducted for the all food isolates revealed significant differences between aerobic and anaerobic conditions. The *p*-value was < 0.0001 against both SA1 and SDG4, while it was slightly higher against SPA1 ($p < 0.0004$). Regarding *Lactobacillus* type strains, significant differences were also obtained between the two conditions of incubation. Anaerobic incubation was significantly higher than the aerobic with *p*-values of 0.01 for SA1 and 0.02 for both SPA1 and SDG4 (Figure 5.7A, B and C). Since the significant differences of food isolates were higher than those of type strains, it could be concluded that food isolates possess an antibacterial potential more than type strains against all the indicator pathogens. Table 5.1 showed the data of the statistical analysis which was performed to compare between the antagonistic activity of all *Lactobacillus* species under the aerobic and anaerobic conditions. The values obtained from the Two-tailed unpaired t-test in GraphPad Prism 8 include: difference between the two means (aerobic and anaerobic), standard error of means, degrees of freedom, t-value.

Figure 5.8 shows the high antibacterial activity of *Lb. plantarum* (Lb23) against SPA1 under anaerobic conditions (Figure 5.8B), compared to the plates incubated aerobically (Figure 5.8A). Regardless of the incubation condition, this figure also indicates the increased antibacterial action after the third day of incubation. Figure 5.9 displays the difference of the antibacterial activity produced by the type strain *Lb. acidophilus* LMG 19170 (Lb26) against SA1 after three days of incubation both anaerobically and aerobically. Diameters of inhibition

zones produced by all *Lactobacillus* bacterial species (including food isolates and the type strains) against each of the three pathogens under both incubation conditions were graphically plotted to explore the isolate or strain with the highest antibacterial effect. Box plots were generated by using BoxPlotR, which is a web-tool for generation of box plots (Spitzer *et al.* 2014). Figures 5.10, 5.11 and 5.12 show the graphs describing the antibacterial activity of all lactobacilli against *S. aureus* (SA1), *S. pyogenes* (SPA1) and *S. dysgalactiae* subsp. *equisimilis* (SDG4), respectively. A red box was drawn around the box plot related to the isolate or strain that has the highest antibacterial potency. Regarding the first pathogen SA1, two food isolates of *Lb. plantarum* showed the maximum growth inhibition: Lb25 (under aerobic conditions, Figure 5.10A) and Lb21 (under anaerobic conditions, Figure 5.10B). Whereas, the type strain *Lb. plantarum* LMG6907 (Lb29) was the most effective strain under both incubation conditions (Figure 5.10C and D). Two other type strains also showed the same activity of Lb29 under anaerobic conditions, these were *Lb. casei* imunitass (Lb2) and *Lb. plantarum* WCFS1 (Lb6) (Figure 5.10D).

With respect to the second pathogen SPA1, the largest inhibition zone was produced by the food isolated *Lb. delbrueckii*: Lb18 under aerobic conditions (Figure 5.11A). While the isolates Lb17, Lb18, Lb19 and Lb23 all showed the highest inhibitory activity under anaerobic conditions (Figure 5.11B). *Lb. brevis* LMG 6906 (Lb28), *Lb. plantarum* LMG6907 (Lb29) and *Lb. paracasei* subsp. *paracasei* LMG7955) (Lb30) were the type strains which exhibited the maximum inhibitory effect under both incubation conditions (Figure 5.11 C and D). In terms of the third pathogen SDG4, the maximum antibacterial activity was obtained by the food-based isolates *Lb. plantarum* (Lb21) under aerobic incubation (Figure 5.12A) and *Lb. delbrueckii* (Lb19) under anaerobic incubation (Figure 5.12B). The type strains that have the maximum inhibitory activity against this pathogen were, *Lb. casei* LMG6904 (Lb27) and *Lb. casei* imunitass (Lb2) when the incubation performed aerobically and anaerobically, respectively.

5.4.1.2 Determination of the Antagonistic Activity of Neutralised *Lactobacillus* Cultures

Despite the cultivation of three *Lactobacillus* isolates (Lb2, Lb25 and Lb29) on buffered MRS agar medium (neutralised cultures), it was observed that these cultures which were incubated for 72h still showed their inhibitory activity against the pathogens. Table 5.2 displays inhibition zone diameters of the pathogenic bacteria by the effect of neutralised cultures of three *Lactobacillus* species. In contrast to the inhibitory effect of these bacterial species cultivated on

non-buffered MRS agar medium (acidic cultures) (Table 5.3), it was observed that the anti-pathogenic effect decreased for most of buffered *Lactobacillus* cultures (7 from the total of 9), when antagonism plates were incubated under anaerobic conditions. While the antibacterial activity increased for a couple of buffered *Lactobacillus* cultures (5 from the total of 9) under aerobic incubation.

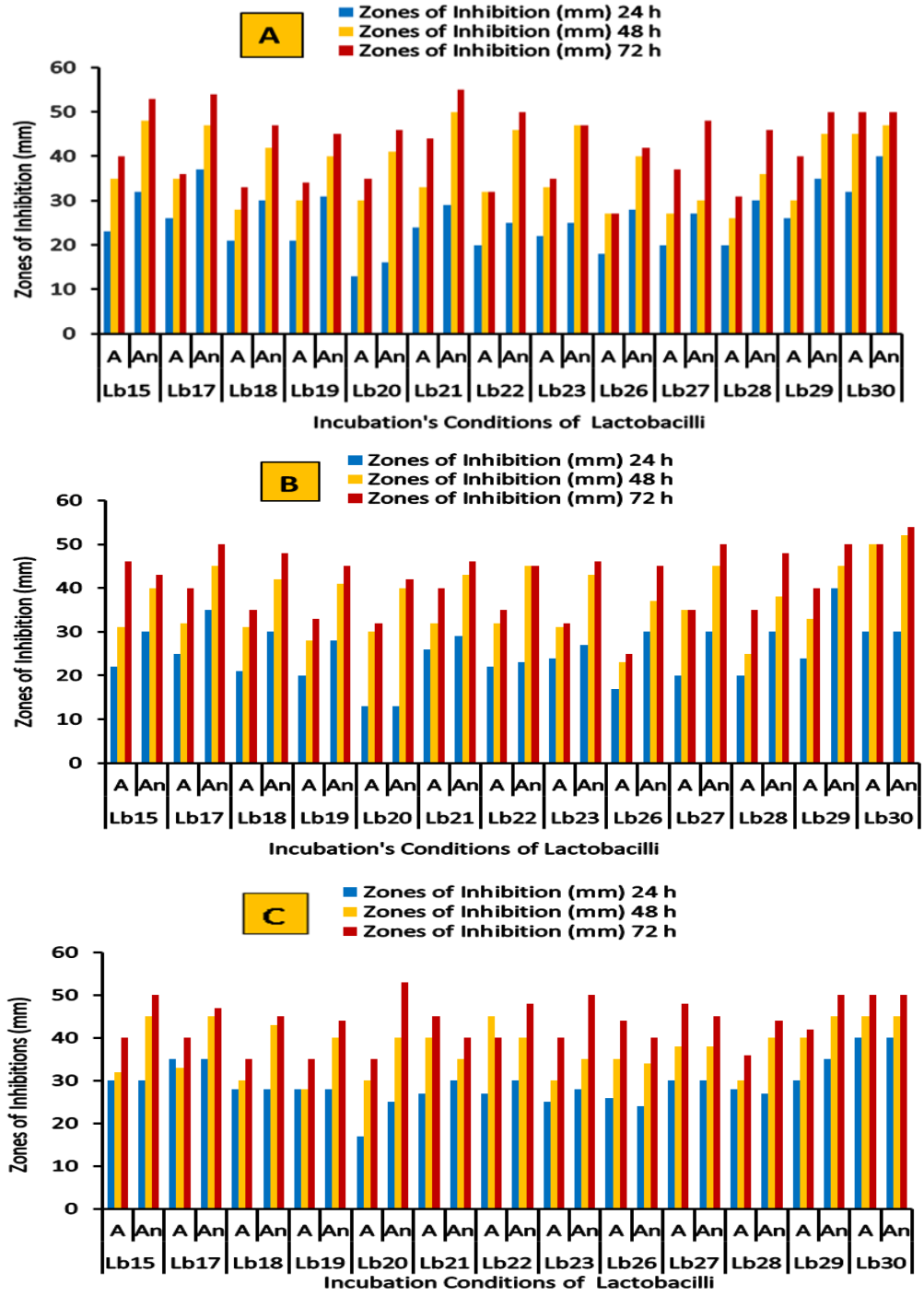


Figure 5.2 Comparison of inhibition zone diameters (mm) of pathogens A: *S. aureus* (SA1), B: *S. pyogenes* (SPA1) and C: *S. dysgalactiae* subsp. *equisimilis* (SDG4) by lactobacilli (Lb15-Lb19: *Lb. delbrueckii*, Lb20-Lb23: *Lb. plantarum*, Lb26: *Lb. acidophilus* LMG19170, Lb27: *Lb. casei* LMG6904, Lb28: *Lb. brevis* LMG 6906, Lb29: *Lb. plantarum* LMG6907, Lb30: *Lb. paracasei* subsp. *paracasei* LMG7955) using overlay method under two different incubation's conditions (A: Aerobic, An: Anaerobic) after 24h, 48h and 72h

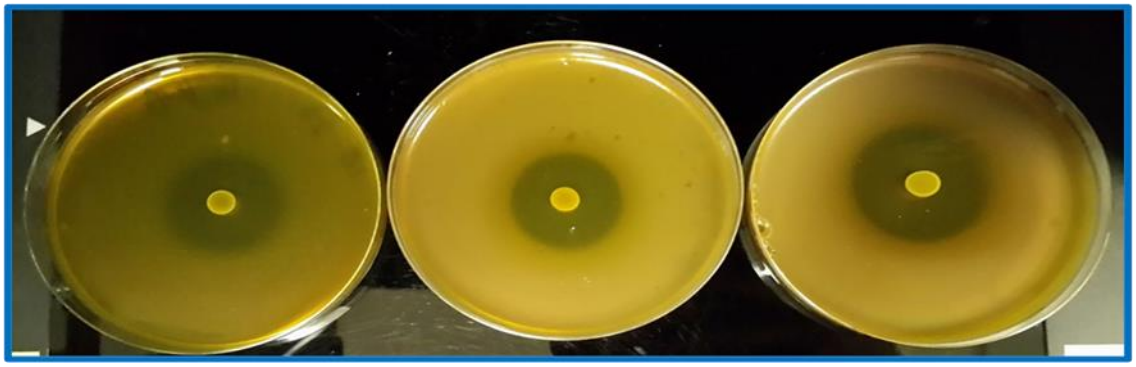


Figure 5.3 Antibacterial activity of the food isolate *Lb. delbrueckii* (Lb18) isolated from yogurt against *S. dysgalactiae* subsp. *equisimilis* (SDG4) by an overlay method under aerobic incubation for three days (from left to right 24h, 48h and 72h

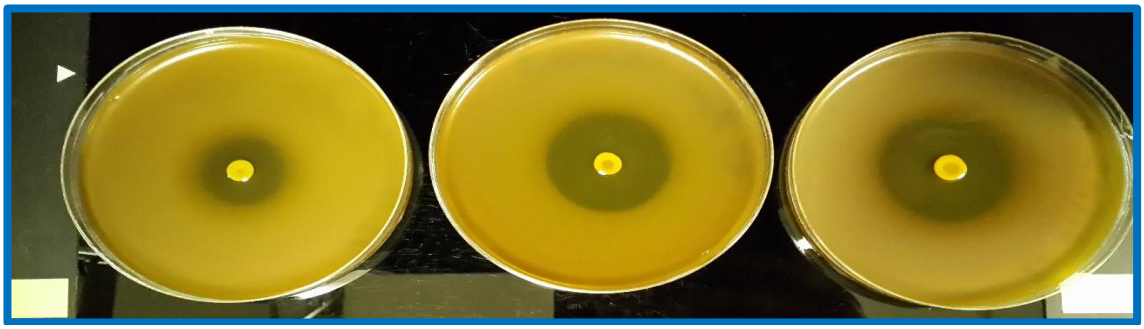


Figure 5.4 Antibacterial activity of the food isolate *Lb. plantarum* (Lb23) isolated from olives against *S. aureus* (SA1) by an overlay method under aerobic incubation for three days (from left to right 24h, 48h and 72h)

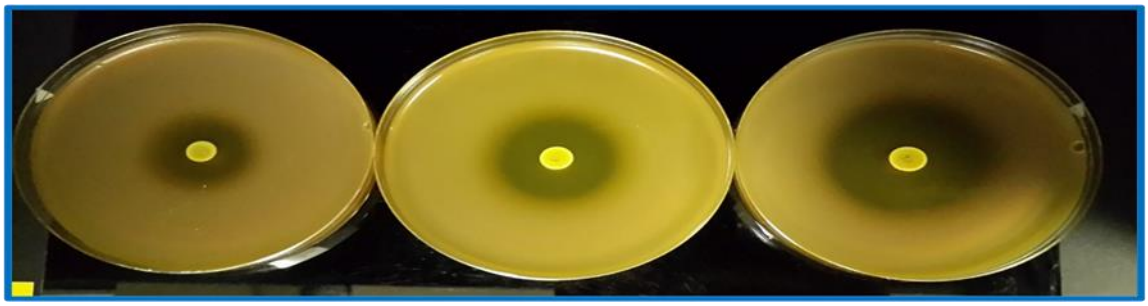


Figure 5.5 Antibacterial activity of the type strain *Lb. casei* LMG 6904 (Lb27) against *S. aureus* (SA1) by an overlay method under aerobic incubation for three days (from left to right 24h, 48h and 72h)

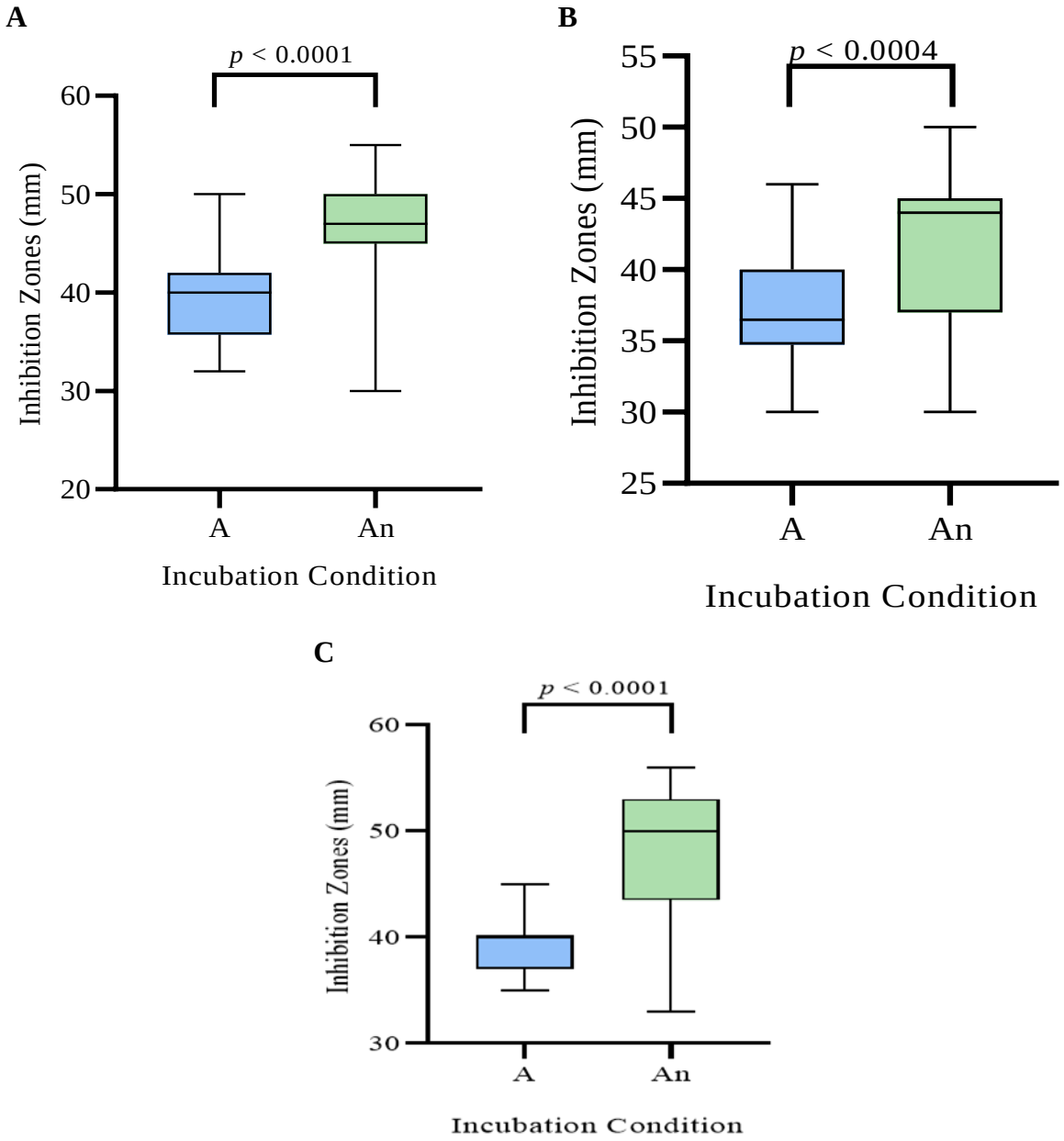


Figure 5.6 Inhibition zone diameters (mm) produced by *Lactobacillus* food isolates against A: *S. aureus* (SA1), B: *S. pyogenes* (SPA1) and C: *S. dysgalactiae* subsp. *equisimilis* (SDG4) under aerobic (A) and anaerobic (An) conditions. Statistical analysis was performed using Two-tailed Unpaired t-test in GraphPad Prism8. Significant differences between the two incubation conditions were calculated with a p-value ≤ 0.05, these values were shown on each figure

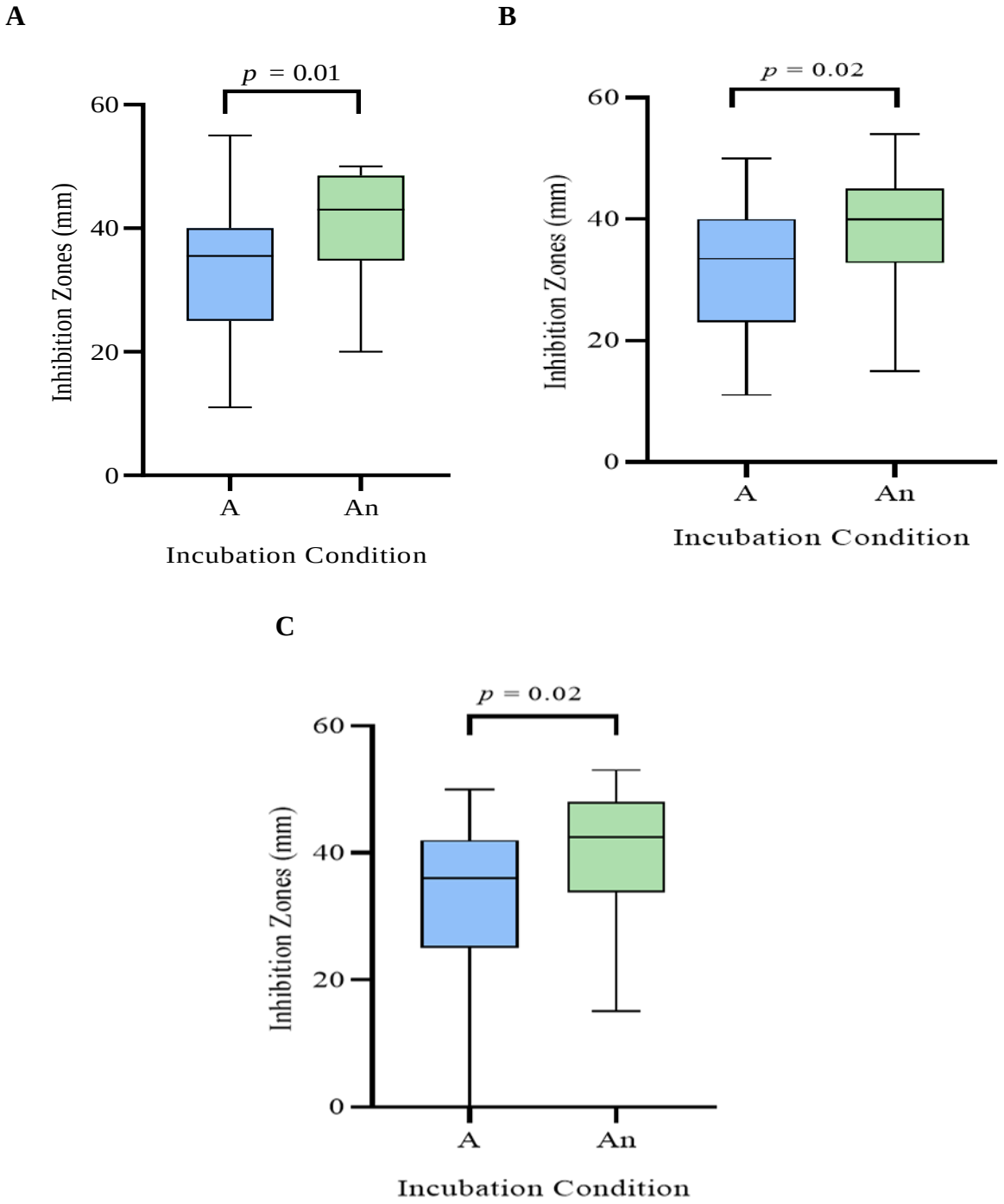


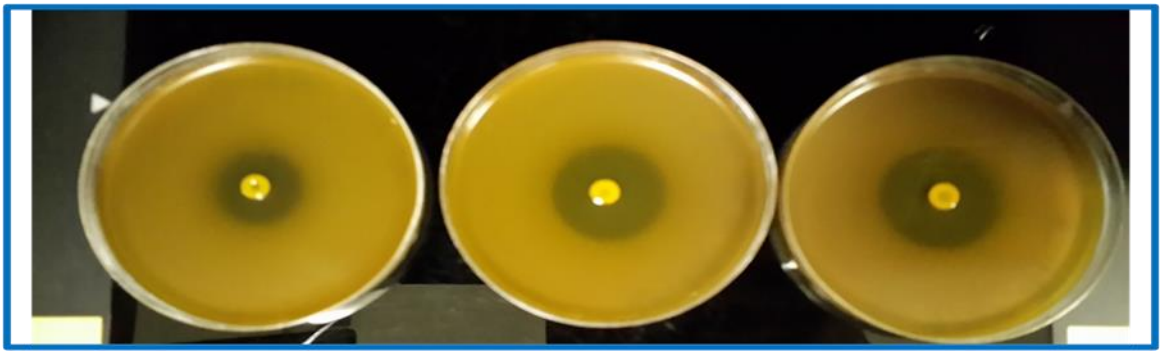
Figure 5.7 Inhibition zone diameters (mm) produced by *Lactobacillus* type strains against A: *S. aureus* (SA1), B: *S. pyogenes* (SPA1) and C: *S. dysgalactiae* subsp. *equisimilis* (SDG4) under aerobic (A) and anaerobic (An) conditions. Statistical analysis was performed using Two-tailed Unpaired t-test in GraphPad Prism8. Significant differences between the two incubation conditions were calculated with a *p*-value ≤ 0.05, these values were shown on each figure

Table 5.1 Statistical analysis data obtained from comparisons between the antagonistic activity of all *Lactobacillus* species under aerobic and anaerobic conditions. Two-tailed unpaired t-test in GraphPad Prism 8 was performed. Statistical analysis values shown in the table include: difference between the two means (aerobic and anaerobic), standard errors of means, degrees of freedom, t-value

Antagonistic Groups Lactobacilli Against Pathogens	**Statistical Analysis Results**		
	Difference between means (A-An) ± Standard Errors of means (SEM)	**Degrees of Freedom (df)**	**t-value**
Food Isolates X SA1	-6.83 ± 1.38	58	4.94
Food Isolates X SPA1	-4.67 ± 1.23	58	3.79
Food Isolates X SDG4	-8.20 ± 1.29	58	6.36
Type Strains X SA1	-7.60 ± 2.64	58	2.88
Type Strains X SPA1	-6.47 ± 2.63	58	2.46
Type Strains X SDG4	-6.97 ± 2.94	58	2.37

SA1: *S. aureus*, SPA1: *S. pyogenes*, SDG4: *S. dysgalactiae* subsp. *equisimilis*

A



B

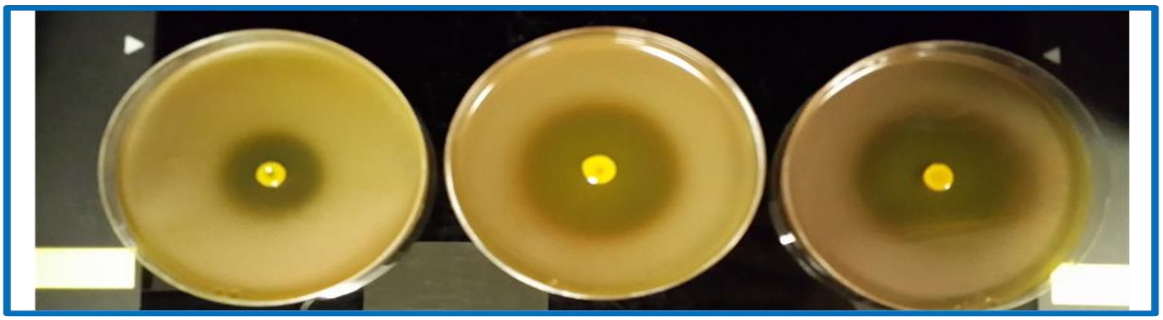


Figure 5.8 Antibacterial activity of the food isolate *Lb. plantarum* (Lb23) isolated from olives against *S. pyogenes* (SPA1) by an overlay method under A: aerobic incubation and B: anaerobic incubation for three days (from left to right 24h, 48h and 72h)

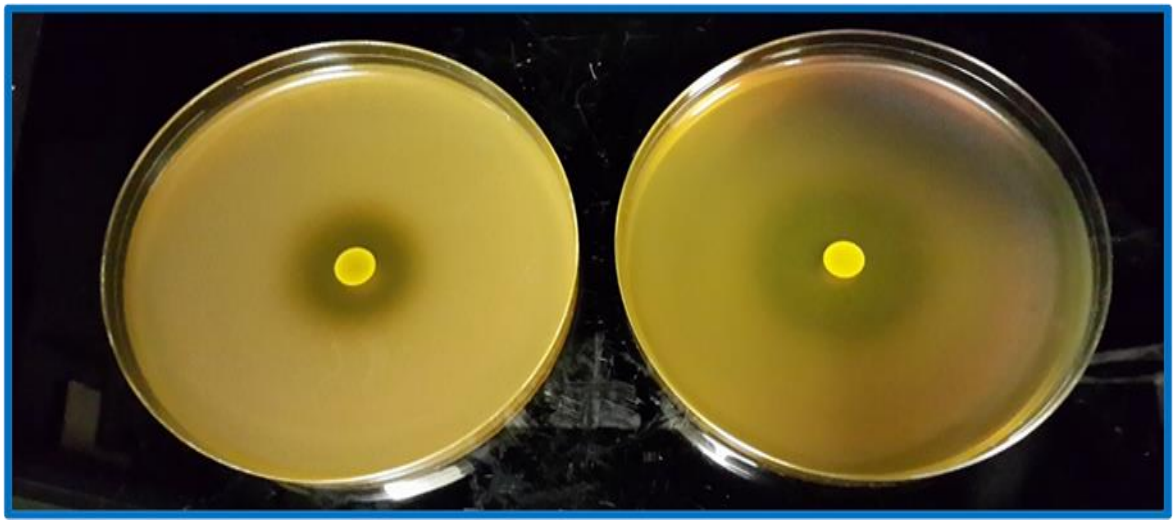


Figure 5.9 Antibacterial activity of the type strain *Lb. acidophilus* LMG 19170 (Lb26) against *S. aureus* (SA1) by an overlay method under two incubation conditions: aerobic (left) and anaerobic (right) after 72h

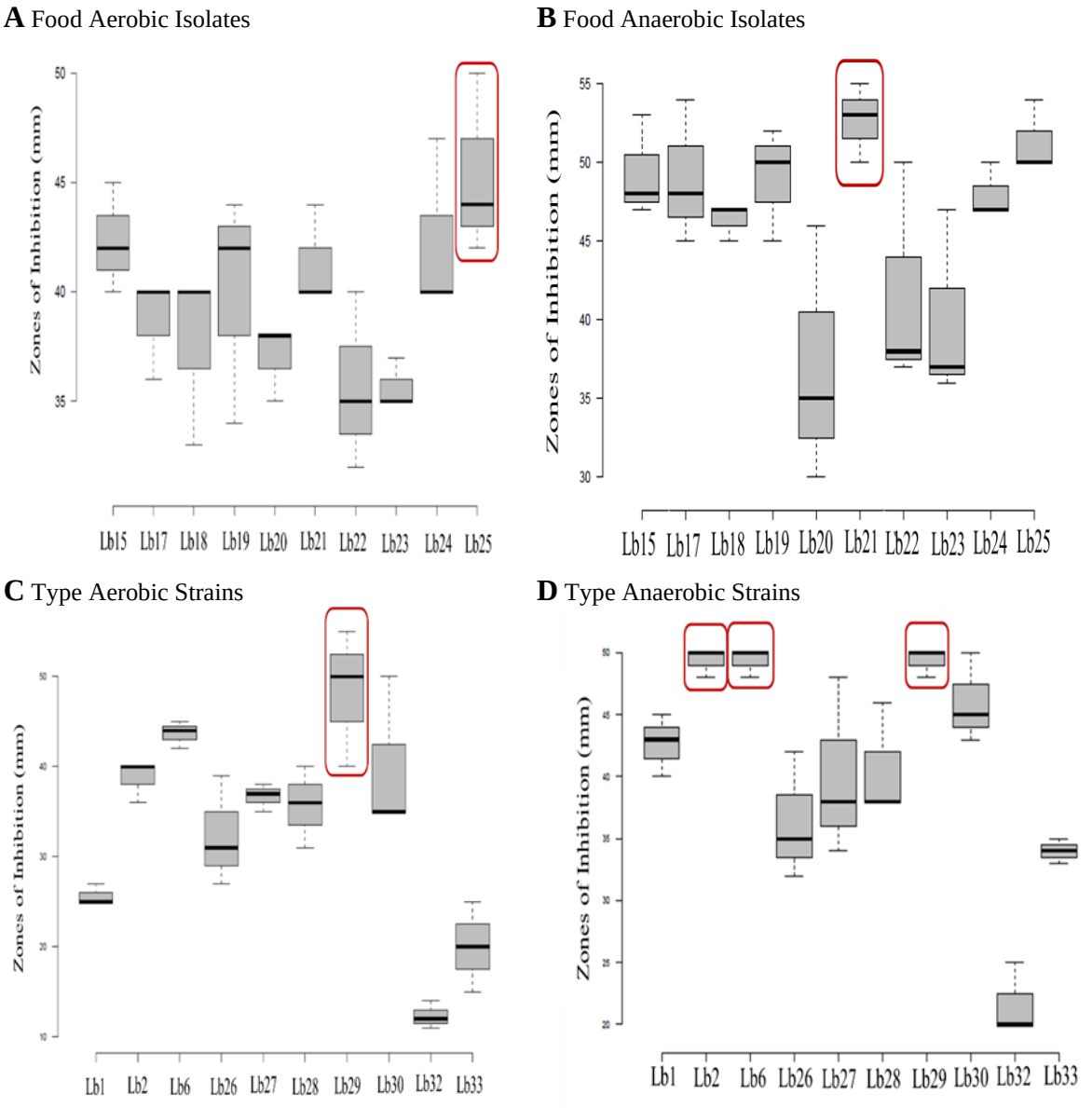


Figure 5.10 Antibacterial activity of lactobacilli food isolates (A and B) and type strains (C and D) on *S. aureus* (SA1) under aerobic (A and C) and anaerobic (B and D) conditions. The red line drawn around the box plot of the food-based isolate or type strain that revealed the highest inhibitory activity against the pathogen.

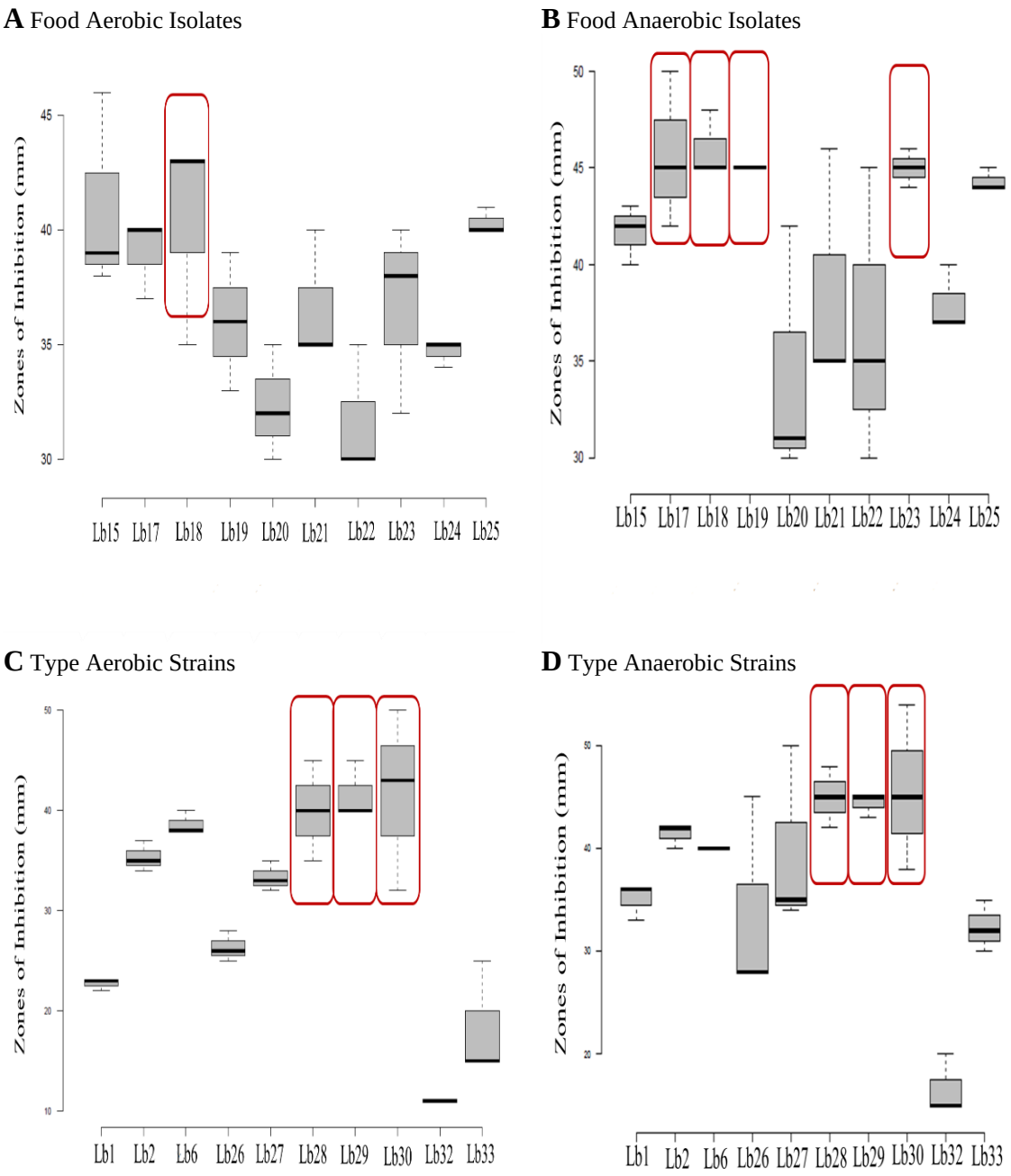


Figure 5.11 Antibacterial activity of lactobacilli food isolates (A and B) and type strains (C and D) on *S. pyogenes* (SPA1) under aerobic (A and C) and anaerobic (B and D) conditions. The red line drawn around the box plot of the food-based isolate or type strain that revealed the highest inhibitory activity against the pathogen.

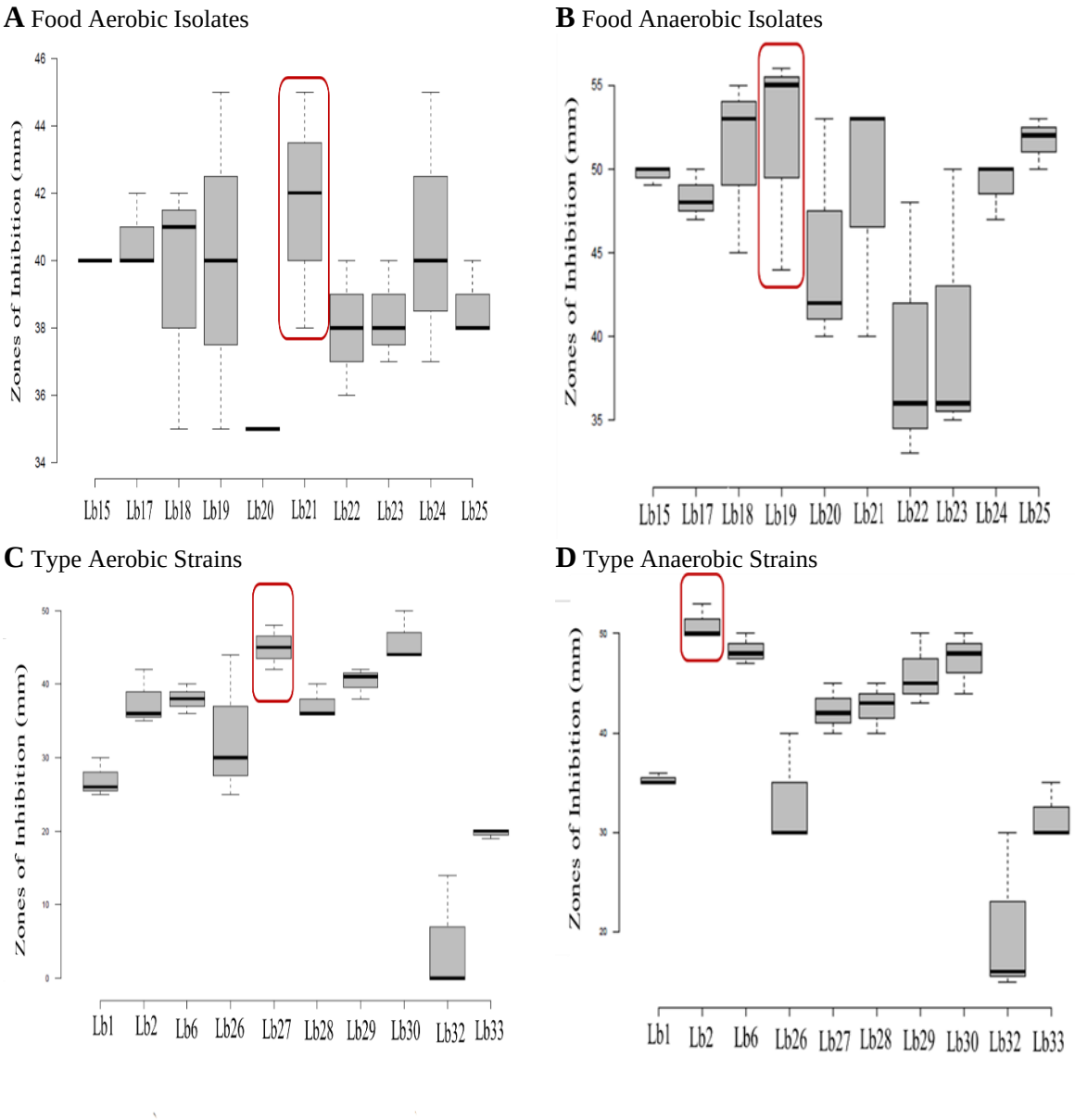


Figure 5.12 Antibacterial activity of lactobacilli food isolates (A and B) and type strains (C and D) on *S. dysgalactiae* subsp. *equisimilis* (SDG4) under aerobic (A and C) and anerobic (B and D) conditions. The red line drawn around the box plot of the food-based isolate or type strain that revealed the highest inhibitory activity against the pathogen.

Table 5.2 Antibacterial activity of *Lactobacillus* cultures cultivated on buffered MRS agar medium and incubated for 72h against the three pathogenic bacterial species, this experiment was conducted once

Lb Species	Buffer Concentration	Incubation Condition	Inhibition Zones (mm)		
			SA1	SPA1	SDG4
Lb2	a	A	60	45	40
		An	45	40	50
	b	A	17	20	15
		An	22	22	23
Lb25	a	A	36	40	42
		An	45	38	50
	b	A	25	23	29
		An	25	23	27
Lb29	a	A	45	40	45
		An	50	40	53
	b	A	21	26	34
		An	28	25	29

Lb: *Lactobacillus*, a: the buffer concentration of 0.01M, b: the buffer concentration of 0.1M, SA1: *S. aureus*, SPA1: *S. pyogenes* and SDG4: *S. dysgalactiae* subsp. *Equisimilis*.

Table 5.3 Antibacterial activity of *Lactobacillus* cultures cultivated on non-buffered MRS agar medium and incubated for 72h against the three pathogenic bacterial species (inhibition zones were taken from Appendices 9 and 10, this experiment was conducted in triplicate)

Lb Species	Incubation Condition	Inhibition Zones (mm)		
		SA1	SPA1	SDG4
Lb2	A	38.7	35.3	37.7
	An	49.3	41.3	51
Lb25	A	45.3	40.3	38.7
	An	51.3	44.3	51.7
Lb29	A	48.3	41.7	40.3
	An	49.3	44.3	46

Lb: *Lactobacillus*, SA1: *S. aureus*, SPA1: *S. pyogenes* and SDG4: *S. dysgalactiae* subsp. *equisimilis*

5.5 Discussion

5.5.1 Mechanisms of Anti-pathogenic Activity of Probiotics

Significant antibacterial activity was observed for all *Lactobacillus* species tested against all pathogenic bacterial species used in this research. Çadirci and Çitak (2005) mentioned the potency of the antimicrobial substances produced by *Lactobacillus* for the inhibition of pathogenic microorganisms. Probiotics can inhibit the growth of pathogens via several mechanisms. One of these mechanisms is the production of antimicrobial compounds including sugar catabolites such as organic acids (e.g., lactic acid and acetic acid); oxygen catabolites such as hydrogen peroxide; proteinaceous compounds such as bacteriocins; fat and amino acid metabolites such as fatty acids, phenyllactic acid and OH-phenyllactic acid; and other compound such as reuterin and reutericyclin. The ability of probiotics to produce these compounds is an example of the functional features used for characterisation of probiotics (Fuller 1989; Makras *et al.* 2006). According to an investigation conducted by Çon and Gökalp (2000), it was reported that bacteriocin-like metabolites produced by lactic acid bacteria during the assay period have an important contribution on the inhibitory activity. Soleimani and co-workers mentioned that lactobacilli have many surface proteins, which are expected to encourage binding to environmental surfaces like other bacteria surfaces (Soleimani *et al.* 2010). Co-aggregation is a phenomenon that would enhance cell to cell closer contact and might be useful for *Lactobacillus* which produce antimicrobial compounds (Reid *et al.* 1988).

Production of organic acids is the mechanism in which probiotic bacteria show their impact on the acidity of the media (Sinclair and Stokes 1962). Shah (2007) mentioned that organic acids (particularly lactic and acetic acids) produced by probiotic bacteria in the gut leads to the decrease of pH which has a bacteriostatic and bactericidal influence. It has been stated by Glass and co-workers that lactic acid has a greater growth inhibitory potency than HCl at an equal pH (Glass *et al.* 1992). Ouwehand and Salminen (1998) reported that the ability of antimicrobial compounds production could be one of the important features for efficient prohibition of pathogen survival in the intestine and appearance of a probiotic influence for the host. Bacterial competition in natural environments is affected by a significant feature which is the ability to produce bacteriocins. Bacteriocins are ribosomally synthesized small antimicrobial peptides which are produced by several different bacterial species and supress the growth of the same or closely related species (Klaenhammer 1988, 1993). There is an extensive investigation

on bacteriocins that have been integrated as a biopreservative component into model food systems. These bacteriocins have an effective impact on the control of spoilage and pathogenic microbes (Neysens and De Vuyst 2005).

Because of the production of bactericidal bioactive peptides (bacteriocins) and enzymes by lactic acid bacterial strains, these bacteria have promising ability to restrict pathogens growth and biofilm formation. *Lactococcus* is one of lactic acid bacterial genera which has been accepted for application in food products (Hansen and Sandine 1994; Millette *et al.* 2007), this genus produces the best defined bacteriocin which is Nisin (Noro and Yang 1995). Massi and colleagues reported four types of LAB strains which have been investigated for their competitive inhibiting characteristics against pathogens (Massi *et al.* 2004). These strains including: *Lb. casei* Shirota, *Lb. casei* 99p rhamnosus GG, *Lb. acidophilus* Johnsonni and *Bifidobacterium brave* Yacult (Nomoto 2005). A number of studies explained the inhibitory activity of lactobacilli against Gram positive and negative bacterial species, as well as multiple beneficial properties of LAB (Nomoto 2005; Maragkoudakis *et al.* 2006). It has been declared by Charlier and co-workers that the growth of *S. aureus* has been inhibited by *Lactococcus lactis* (Charlier et al. 2008). Several investigations have been conducted anticipating that LAB species have the ability to inhibit *S. aureus* by the production of hydrogen peroxide, bacteriocins and organic acids like acetic and lactic acid (Hernandez *et al.* 2005; Lin *et al.* 2006; Charlier *et al.* 2008). Millette and colleagues confirmed that the whey isolated from milk fermentation transformed by strains CL1285® *Lb. acidophilus* and *Lb. casei* LBC80R has the ability to inhibit the pathogenic bacteria including: Methicillin sensitive *S. aureus* (MSSA), *Listeria monocytogenes* and *E. coli* 0157:H by inhibitory percentages of 85%, 78% and 77%, respectively. Furthermore, the growth of these food-borne pathogens has been delayed by the bacterial cultures of these *Lactobacillus* species (Millette *et al.* 2007).

Despite the acknowledged beneficial effects which can be exerted by certain probiotics, there is limited information about mode of actions underlying these effects. There is a close relationship between these mechanisms and the properties, manufacturing and formulation of the selected probiotic strains (Grześkowiak *et al.* 2011). However, the well-known mechanisms for all strains include: elimination of pathogen colonisation by competition with pathogenic bacteria for adhesion sites and/or nutrients and growth factors (Lu and Walker 2001; Zhang *et al.* 2010); production of organic acids which decrease the pH and inhibit the growth of pathogens (Yamano *et al.* 2006). Production of inhibitory compounds such as bacteriocins and other toxic primary metabolites destructive to pathogens (Nemcova 1997; Kopp-Hoolihan 2001).

Modulation of the host immune system (Kailasapathy and Chin 2000; Zhang *et al.* 2010) and suppression of bacterial toxins (Corthier *et al.* 1985).

5.5.2 Experimental Factors Affecting the Antagonistic Activity

5.5.2.1 The Incubation Time of *Lactobacillus* Species Cultures

The experiment which was performed to assess the antagonistic activity of thirteen *Lactobacillus* species (including food isolates and type strains) against all three pathogens showed various diameters of inhibition zones during the days of incubation. However, it has been detected that all bacterial species revealed a gradual increase of the antibacterial activity approaching the highest potential when the antagonism plates were incubated for three days, regardless of the source of *Lactobacillus* species. The maximum antagonistic effect obtained after 72h of incubation, results from the high number of growing LAB isolate bacterial cells which leads to an increase in the production of antibacterial compounds. It was reported that there is a direct relationship between bacteriocin production and the bacterial growth (Møretrø *et al.* 2000; Calderon-Santoyo *et al.* 2001; Delgado *et al.* 2007). Furthermore, during the bacterial growth, most bacteriocins are produced as primary metabolites (De Vuyst *et al.* 1996; Klostermaier *et al.* 1999; Møretrø *et al.* 2000). Therefore, incubation for a few days could have caused an accumulation of primary metabolites which were produced at the time of logarithmic phase of bacterial growth (first days of bacterial incubation) and production of other secondary metabolites during the stationary phase of the bacterial cell growth. Both of primary and secondary metabolites efficiently contribute and cause the maximum antibacterial activity after three days of incubation.

5.5.2.2 Incubation Conditions of the Antagonism Cultures

Regardless of the source of species (food isolates or type strains), it has been shown that the incubation of antagonism plates under anaerobic condition revealed larger growth inhibition zones than the aerobic condition. This result could be due to two reasons: firstly, *Lactobacillus* species are better adapted to grow under anaerobic conditions. These conditions enhance the bacterial growth and resulted in higher numbers of bacterial cells and their metabolites. More growing bacterial cells lead to more antibacterial bioactive compounds produced in the medium, and high inhibition rate of pathogenic growth. Secondly, the pathogenic species included in our study are facultative anaerobes and grow more under aerobic conditions, so incubation of the

antagonism plates under anaerobic conditions would reduce the growth of these pathogens and resulted in fast growth inhibition and large inhibition zones.

5.5.2.3 The Source of *Lactobacillus* Species

The inhibition of all pathogenic species by food isolates was extremely high compared to that obtained by type strains. This difference was confirmed by the statistical analysis of inhibition zone diameters produced by all *Lactobacillus* species under both aerobic and anaerobic conditions. Regardless of the source of *Lactobacillus* species, statistical analysis showed significant differences between the two incubation conditions. Food isolated lactobacilli have many valuable characteristics which distinguish them from type strains. Firstly, lactobacilli which are in food have been incorporated into eating habits since they are considered generally regarded as safe (GRAS) bacteria (Nomoto 2005). Secondly, due to their recognized probiotic activities, *Lactobacillus* species are usually added to a number of food products. In terms of bacterial type strains existed in culture collections, these are usually kept as freeze-dried preparations for many years since their isolation from their sources. Thus, antimicrobial potency of bacterial species and other physiological or genomic characters could be reduced by long-term storage of bacterial species in culture collections. Furthermore, repeated culturing procedures of bacteria outside their normal isolation sources may also weaken the viability and their capacity to supress the growth of other bacterial species. A similar situation has been shown in Bifidobacteria, which will reduce their genomes when cultured in laboratory media (Lee *et al.* 2008; Lee and O'Sullivan 2010).

It has been stated by Reuter (1969) that gastrointestinal (GI) disorders could be treated by freeze-dried bifidobacterial preparations, sometimes with added *Lb. acidophilus*. During the last century, several authors suggested bifidobacteria as dietary supplements because of the promising health advantages of these bacteria (Rasic and Kurmann 1983; Saavedra and Tschernia 2002; Parvez *et al.* 2006). As we found that the inhibitory activity of type strains lactobacilli is lower than that of food-based lactobacilli, this is in agreement with Lee and co-workers who studied the performance of a specific strain of *B. longum* subsp. *longum* which was cultured several times outside the gut. The research group observed that throughout the culture growth in new environments, this strain has a tendency to undergo genome reduction in regions which are no longer required for the these environments (Lee *et al.* 2008). Regarding *Lactobacillus* species which have been isolated from the traditional natural-fermented dairy

products, several studies on these species indicated that they are safely used for a long time (Holzapfel *et al.* 2001).

5.5.2.4 The pH of *Lactobacillus* Culture Medium

It was found that the inhibitory activity of the three *Lactobacillus* species against the pathogens has persisted, even when these species were cultivated on buffered medium. The remained activity might be due to the inability to achieve the total neutralisation of acid effects, or it could be resulted from other bioactive compounds rather than organic acids which are produced by *Lactobacillus* cultures such as bacteriocins. Both lactobacilli and lactococci species produce bacteriocins such as; lactacin B or F produced by *Lb. acidophilus* and casein 80 produced by *Lb. casei* B80 (Rammelsberg and Radler 1990; Klaenhammer 1993; Karska-Wysocki *et al.* 2010), while Nisin is produced by the genus *Lactococcus* (Noro and Yang 1995; Karska-Wysocki *et al.* 2010). Table 5.4 illustrates the most important bacteriocins produced by lactobacilli. It was mentioned by Jack and co-workers who reported that acidic conditions in the range of 2.0-6.0 are the best environment for the bactericidal activity of bacteriocins produced by Gram positive bacteria (Jack *et al.* 1995). It was also declared by Gänzle and colleagues that the action of antimicrobial compounds may be improved by the influence of acidic conditions in the stomach (Gänzle *et al.* 1999). The pH range of 4.5-5.5 is the most appropriate range for the maximum production of bacteriocins (Krier *et al.* 1998; Klostermaier *et al.* 1999; Calderon-Santoyo *et al.* 2001). Nevertheless, the inhibitory potential of probiotics may not only be caused by the high acidity but there is the potential for bacteriocins too, to be involved.

Results obtained from this experiment confirmed the persistence of the inhibitory potency after cultivation of *Lactobacillus* species on buffered medium. A previous study conducted by Miyazaki and his group also led to the confirmation of our research findings. Miyazaki's group stated that despite of its weak acidic nature (pH 6.4), the supernatant of the probiotic bacterium *Enterococcus faecium* revealed a significant bactericidal activity on enteroaggregative *Escherichia coli* (EAggEC). Following the adjustment of the supernatant pH to 7.0, the bactericidal activity was also detected by this research group. This finding proposed that a bacteriocin produced by *E. faecium* might be the reason behind the bacterial potential against EAggEC (Miyazaki *et al.* 2010). Karska-Wysocki and assistants stated that no relation has been clearly detected between the antimicrobial potential of LAB strains and the acidification of medium (Karska-Wysocki *et al.* 2010). Other investigation carried out by

Maragkoudakis and associates was not in agreement with our results. This research group found that cultivation of pathogens in the presence of nearly neutral supernatants (pH 6.5) revealed no growth inhibition of these pathogens. Thus, they concluded that the inhibition activity was mostly because of the produced organic acids along with the low pH and bacteriocins were not contributed in the antibacterial effect (Maragkoudakis *et al.* 2006).

Table 5.4 Most important bacteriocins produced by lactobacilli (Zacharof and Lovitt 2012)

Bacteriocin	Bacteriocin Producing Strain
Lactacin F	*Lb. johnsonii* spp.
Lactocin 705	*Lb. casei* spp.
Lactoccin G	*Lb. lactis* spp.
Lactococcin MN	*Lactococcus lactis* var *cremoris*
Nisin	*Lactococcus lactis* spp.
Leucocin H	*Leuconostoc* spp.
Plantaricin EF, Plantaricin W Plantaricin JK, Plantaricin S	*Lb. plantarum* spp

Our research findings have detected a decrease in the inhibitory activity of *Lactobacillus* buffered cultures when compared to that of the non-buffered cultures under anaerobic incubation conditions. This might be because the inhibitory activity of non-buffered cultures was due to the antibacterial potential of both organic acids and bacteriocins. whereas the activity of buffered cultures resulted from the effect of bacteriocins alone. In terms of the aerobic conditions, the inhibitory activity increased for a couple of the antagonism experiments. The reason of this increase might be as a result of the high growth of *Lactobacillus* species aerobically, which leads to the increase of the bacteriocin production. Furthermore, the antibacterial potency of some active compounds, like bacteriocins, could be increased when the incubation has been performed aerobically.

The results obtained from our recent investigation is not in agreement with Argyri and colleagues who evaluated the antimicrobial potential of a group of lactic acid bacterial species isolated from naturally fermented table olives. Those species including both *Lactobacillus* and *Leuconostoc* species. This research group found that none of the bacterial supernatants adjusted to pH 6.5 could inhibit the growth of the target pathogens, so they assumed that no existence of a bacteriocin-like action (Argyri *et al.* 2013). Several studies also confirmed that the inhibitory activity of other probiotics turned into insignificant in neutral pH 7.0 such as *Lb. fermentum* strains (Lin *et al.* 2007) or *Lb. casei* Shirota, *Lb. paracasei* subsp. *tolerans* and *Lb. plantarum* (Maragkoudakis *et al.* 2006). Furthermore, it was stated by Millette and co-workers that

neutralisation of the soluble fraction to pH 6.5 resulted in a remarkable reduction in the antimicrobial potency against all the selected pathogens (Millette *et al.* 2007).

5.6 Conclusions

1- The three pathogens used in this study were all sensitive to the antibacterial activity of Lactobacillus species, including food isolates and type strains which revealed different diameters of growth inhibition zones.

2- Regardless of the incubation conditions, *Lactobacillus* species presented a remarkable inhibitory effect after one-day incubation, which gradually increased through the days of incubation, approaching the maximum activity after 72h.

3- Most *Lactobacillus* species revealed higher antibacterial effect against the pathogens under anaerobic conditions more than that obtained aerobically.

4- Food isolated *Lactobacillus* species have higher inhibitory potency than *Lactobacillus* type strains against all the pathogens especially when the antagonism plates were incubated anaerobically. This was concluded by observation of significant differences (obtained from the statistical analysis) between the inhibitory activity under aerobic and anaerobic incubation conditions. These significant differences were higher for food isolates than those for type strains.

5- The *in vitro* antibacterial potency of LAB species proved in this chapter has promising practical applications for *in vivo* investigations. Food-based lactobacilli are especially designated as useful candidates for further exploration. For instance, assessment of their industrial features to be applied as new probiotic starters in fermentation studies. Moreover, enlightening of their valuable health advantages within *in vivo* studies (which has been already performed for two isolates: Lb19 and Lb21 in chapter 6).

6- *Lactobacillus s*pecies cultivated on buffered MRS medium for 72h showed a persistent antibacterial potential against pathogens. This indicates that the activity was not only because of organic acids production, but due to other antibacterial substances such as bacteriocins. Furthermore, buffered bacterial cultures revealed low inhibitory activity when compared to that obtained by non-buffered cultures, especially when the experiment was carried out under anaerobic incubation conditions.

5.7 References

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Chapter Six

Susceptibility of the Greater Wax Moth Larvae *Galleria mellonella* to Bacterial Isolates (*in vivo*)

6 Susceptibility of the Greater Wax Moth Larvae *Galleria mellonella* to Bacterial Isolates (*in Vivo*)

6.1 Introduction

Antibacterial activity of the food *Lactobacillus* species isolated in this study, against the skin pathogens was assessed by using *in vitro* overlay assay in the previous chapter (Chapter 5). To evaluate the therapeutic activity of these species against pathogenic infections, two isolates were selected: *Lb. delbreuckii* (Lb19) isolated from yogurt and *Lb. plantarum* (Lb21) isolated from black olive. Assessment of the therapeutic ability of the selected food-based *Lactobacillus* species was carried out by injection of the two species inside an *in vivo* experimental model *Galleria mellonella* (the greater wax moth), previously infected with the pathogenic bacteria used in this research. The injected larval groups were monitored, and survival percentages were calculated and compared with those of the control groups injected with either *Lactobacillus* isolates or pathogenic species.

Pathogenic species used in this study can cause serious infectious skin diseases with high rates of morbidity and mortality. These bacterial pathogens may develop resistance mechanisms to the available antibacterial medicines, which also have several toxic side effects for the human body. Thus, the development of new therapeutic approaches for the treatment of bacterial infections, becomes important to conquer the problem of multi-drug resistant pathogens. One of the developed therapeutic strategies is the use of bacteria in the treatment of infections which is named “bacterial therapy or bacteriotherapy” (Lee *et al.* 2001; Felgner *et al.* 2016). It is considered as an alternative and promising approach of modern therapy which uses harmless organisms to replace pathogenic bacteria. The presence of these two bacteria within the infection site result in bacterial interference. The concept of bacterial interference is that one organism has the ability to produce compounds which interfere with the virulence factors of the other organism resulting in the suppression of these factors and occurrence of infection healing (Huovinen 2001).

Among the bacteria used for therapy, a lot of interest has been taken in probiotic bacteria. As probiotic bacteria have been introduced into dairy products for many years and consumed with infrequent harmful effects, therefore the safe use of these bacteria has been confirmed to

investigators (Saxelin *et al.* 1996). Many studies reported the effectiveness of probiotic bacteria in the prevention and treatment of some diseases such as (Nguyen *et al.* 2007; Moayyedi *et al.* 2008; Schreck Bird *et al.* 2017; Wallace and Milev 2017). Treatment of skin diseases has been successfully achieved by the use of probiotics (Yeşilova *et al.* 2012; Notay *et al.* 2017). Furthermore, it was stated that some strains of probiotic bacteria displayed several advantages in the process of wound healing (El-Ghazely *et al.* 2016; Shahsafi 2017). However, the beneficial effects of probiotics on skin conditions have been demonstrated by the oral administration of these bacteria as tablets or capsules. The topical application of probiotic bacteria for the treatment of skin problems, was rarely studied and is increasingly being investigated (Al-Ghazzewi and Tester 2014).

It has been shown by previous studies that probiotic bacteria have a significant function in the prevention of local infections. A patent performed by Hansen and Jespersen stated that the growth and colonization of pathogenic bacteria existed on the wound surface could be inhibited by probiotic lactic acid bacteria through different mechanisms, resulting in acceleration of the wound healing process (Hansen and Jespersen 2010). Furthermore, *Lactobacillus reuteri* has been used in another investigation as a topical application and proved its capacity to prevent the opportunistic bacteria colonization and wounds infection (Prince *et al.* 2012). In addition, it was reported that *Lb. acidophilus*-derived surfactants had an inhibitory effect on the adhesion and biofilm formation of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which are the main opportunistic bacteria causing wound and burn infections (Walencka *et al.* 2008). Jebur (2010) stated the antibacterial activity of *Lb. acidophilus* 1x 10^8 cell/ml on the growth of burn and wound causatives including *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* obtained from patients in Baghdad hospitals. Barzegari and co-workers detected the potency of *Lb. acidophilus* in the prevention of wounds infection in rats, as no signs of infected wounds were observed indicating that the growth of pathogenic bacteria was prohibited due to the antibacterial activity of this species. It was also found that *Lb. acidophilus* revealed other beneficial impacts during different stages of wound healing such as reduction of the inflammatory response and increase of rate of the granulation tissue formation and re-epithelialization (Barzegari *et al.* 2017). In another study conducted by Mohammedsaeed and colleagues who reported the ability of *Lactobacillus rhamnosus* GG in increasing the re-epithelialization in a keratinocyte scratch investigation, by the production of lysate which stimulates the migration of keratinocytes (Mohammedsaeed *et al.* 2015). It was also stated that

Lactobacillus live cells, lysate, and conditioned media were effective in protecting the epidermal keratinocytes from the toxic effect of *S. aureus* (Mohammedsaeed *et al.* 2014).

Lactobacillus isolates with probiotic characteristics have established promising therapies for infections. These features include the adherence to mucosal epithelial cells of the host (Martin *et al.* 2008; Nishiyama *et al.* 2013), suppression of pathogens colonization and binding to the mucus and epithelial cells (Castro *et al.* 2013), repression of the virulence factors produced by pathogens (Hugo *et al.* 2008), modulation of the host immune response (Ohland and MacNaughton 2010; Aoudia *et al.* 2016) and possessing an antimicrobial efficiency (Martin *et al.* 2008; Bermudez-Brito *et al.* 2012). Probiotic bacteria have the ability to produce antibacterial substances which prevent or inhibit the spread of pathogens, such as bacteriocins, hydrogen peroxide and lactic acid (Bermudez-Brito *et al.* 2012; Aoudia *et al.* 2016). The inhibition of pathogens may be caused by the independent involvement of one of these antibacterial substances, or by the combined effect of all of them (Lebeer *et al.* 2008).

The valuable features of probiotic bacteria have been stated in a large number of scientific studies by performing experiments using mammalian hosts such as mice. The mouse model has been mainly used as an *in vivo* tool since it shows a similar immune response to that of the human and sometimes similar sensitivity to microbes. Nevertheless, investigations on microbial pathogenesis are conducted by using invertebrates instead of mammalian hosts as many of their physiological characteristics are evolutionary conserved. Furthermore, they are easily obtained, economically preferable without the involvement of ethical issues required for mammalian studies (Glavis-Bloom *et al.* 2012). One of the most widely spreadable groups of animals on earth are insects. Their topographical distribution is broad since they are able to inhabit within large numbers of environments which are difficult to be occupied by other life forms of animals. The estimated number of insect species is approximately 750,000-1,000,000 species, therefore, they are considered as the most varied forms of animals (Ratcliffe 1985). It has been recognized that the innate immune system of insects is similar to that of mammals. Both immune systems share functional homology and have both humoral and cellular immunity. Distinguishing the insect response to infection could be helpful in the determination of useful information associated with the function of the mammalian innate system (Hoffmann 1995; Salzet 2001; Lionakis 2011). An insect's cuticle plays the same role as that of skin in mammals, it functions as a first barrier to protect insects against pathogens. The cuticle has a complicated chemical and structural composition which helps in prevention of pathogens entry into the haemoceol (the body cavity) (Clarkson *et al.* 1998). The insect haemoceol contains haemolymph

which is analogous to blood in mammals, it helps in transporting of nutrients and waste products (Matha and Áček 1984). Furthermore, haemolymph includes cells and antimicrobial peptides that have the ability to restrict and kill invading microbes (Morton *et al.* 1987; Vilmos and Kurucz 1998). Haemocytes are also present in the insect haemolymph; they have a similar function of phagocytic cells in mammals and are able to engulf pathogenic bacteria and kill them by respiratory burst (Bergin *et al.* 2005). The majority of haemocytes moves easily within the haemolymph, however a considerable number (up to 30% in some insect species) can be associated with internal organs such as digestive system or trachea (Ratcliffe 1985). Recently, the larvae of the wax moth *Galleria mellonella* (also known as the wax worm) has been well acknowledged by scientists as an *in vivo* experimental model for different bacterial and fungal infections (García-Rodas *et al.* 2011; Junqueira 2012; Arvanitis *et al.* 2013; Alghoribi *et al.* 2014). Valuable information about pathogenesis, virulence mechanisms and antimicrobial efficacy can be provided by using this model (García-Rodas *et al.* 2011; Mesa-Arango *et al.* 2013; de Lacorte Singulani *et al.* 2016). Since there is a similarity in the microbial virulence in the wax worm and mammals, the virulence of various human pathogens could be investigated in *G. mellonella* larvae (Champion *et al.* 2009; Peleg *et al.* 2009a; Gao *et al.* 2010; Mukherjee *et al.* 2010).

It has been found by a number of studies performed in the last few years that the results acquired from *G. mellonella* and mammalian models are positively correlated (Brunke *et al.* 2015; Sangalli-Leite *et al.* 2016). The insect model *G. mellonella* has many advantages that other non-mammalian models lack, such as the ability to grow at temperatures range between 25-37°C, low cost, large-rate of breeding, easy and rapid test performance and the conservative evolution compared to mammals (Mylonakis 2008; Arvanitis *et al.* 2013). Moreover, there is a close correlation between this model and humans in some aspects. The insect immune response is similar to the human innate immune response, and they both have similar mechanisms for killing the bacterial or fungal pathogens (García-Lara *et al.* 2005; Nathan 2014). Furthermore, a precise inoculum of the pathogen can be easily delivered into the larvae body by numerous available routes (Fuchs *et al.* 2010). The injection of a consistent microbial inoculum is the infection technique which has been introduced by a large number of studies associated with the wax worm (Jorjão *et al.* 2018). Nevertheless, infection can also be conducted through oral delivery or ingestion (Fedhila *et al.* 2010). In addition to these benefits, *G. mellonella* can be used for evaluating the antimicrobial agents efficiency, and this model has already been used to

investigate therapies of the infections caused by *Acinetobacter baumannii* and fungi (Peleg *et al.* 2009a; Rowan *et al.* 2009).

A continuous emphasis was undertaken by the biomedical research funding organisations on robust and rapid animal investigations. Animal modelling showed a progress towards using models that most properly resemble human conditions. Moreover, multiple models were required for the confirmation of data robustness. Currently, with the expansion of novel genetic and genomic tools, genetic engineering strategies can be efficiently applied to develop and improve "humanized models" such as transgenic animals which are used in research. This includes either insertion of specific human genes or implanting a certain human tissue into animals achieving the status named as "humanization" in which the graft of human cells presents their primary functions in the recipient animal. As a result, researchers could explore responses to the pathogenic infection as if it were occurring in human environment (Stoltz *et al.* 2010; Ericsson *et al.* 2013; Ernst 2016).

6.2 Aims

1- To investigate the susceptibility of the *in vivo* model, *G. mellonella* larvae to the injection effect of different treatments (bacterial suspensions, washed cells and supernatants) of ten food isolated *Lactobacillus* species: four *Lb. delbreuckii* and six *Lb. plantarum*. Furthermore, to determine the injected bacterial dose which reveals the highest percentage of larvae survived.

2- To investigate the susceptibility of *G. mellonella* larvae to the injection effect of different treatments (bacterial suspensions and washed cells) of three skin pathogens: *S. aureus*, *S. pyogenes* and *S. dysgalactiae* subsp. *equisimilis*. Moreover, to determine the injected bacterial dose which has the highest lethal effect on the larvae and the dose which kills 50% of the larvae (LD_{50} value).

3- To explore the therapeutic potency of two selected food *Lactobacillus* species: *Lb. delbreuckii* (Lb19) and *Lb. plantarum* (Lb21) against the infections caused by skin pathogens in *G. mellonella* larvae by the performance of co-injection (challenge) experiments, in which several doses of pathogenic species were injected with two different doses of *Lactobacillus* species.

4- To evaluate the efficiency of the topical treatment of skin infections with the food-based *Lactobacillus* species, by the topical application of *Lactobacillus* bacterial cells to the body surface of the larvae previously infected with the pathogen.

6.3 Methods

6.3.1 Classical Infection of *G. mellonella* Larvae (Injection)

6.3.1.1 Galleria mellonella Injection Method

Final instar larvae of the greater wax moth *G. mellonella* weighing 0.18-0.35 g were purchased from Bio Systems Technology Ltd (Exeter, UK) packed in sterilised vented plastic containers, kept at room temperature in the dark and used within seven days of delivery. The susceptibility of the larvae to food *Lactobacillus* species was tested by injection with 50 μL Hamilton micro syringes and 25G syringe needles. For each treatment and control group, ten larvae were randomly selected and placed in Petri dishes. An individual larva was injected with 10 μl of each dose of *Lactobacillus* bacterial suspension, supernatant and washed cells, the injection was done into the hemocoel through the last left proleg. The same amount 10 μl of each individual dose of pathogenic bacterial suspensions and washed cells was also injected inside the larvae. Following injection, larvae were placed in sterile petri plates, incubated in the dark at 37°C and the number of dead larvae was counted and recorded daily for three days. Control groups injected with 10 μl of 1x PBS (0.01 M) were included in the experiment. Larvae were considered dead when they did not respond or move on stimulation of touch with forceps (Ramarao *et al.* 2012). For all injection assays, survival curves were generated during the three incubation days.

6.3.1.2 Preparation of the Injected Bacterial Solutions

To evaluate the susceptibility of the wax moth larvae *G. mellonella* to the bacterial species, the ten food isolates of *Lactobacillus* (four *Lb. delbrueckii* and six *Lb. plantarum*) were each inoculated in 5 ml MRS broth medium, one day before the injection experiment and incubated anaerobically at 37°C for 18 h. The bacterial suspension of each isolate was centrifuged at 2000 g for 10 min at 4°C. The obtained supernatant was transferred into a new tube and sterilised twice by 0.25µ Millipore filters. Whereas the pellet was washed four times by using 1x (0.01M) phosphate buffered saline (PBS), pH 7.4 (Severn Biotechnology), and re-suspended in the saline to be used as a washed cells solution. Several decimal dilutions were prepared from each of bacterial suspensions, supernatants and washed cells by using the same concentration of PBS, these dilutions were injected later in the larvae.

In terms of pathogenic bacterial species, each of the three pathogens (*S. aureus*, *S. pyogenes* and *S. dysgalactiae* subsp. *equisimilis*) was grown in 5 ml TS broth medium and incubated under aerobic conditions at 37°C for 18 h. On the next day, bacterial cells were prepared from bacterial culture suspensions by following the same steps performed for *Lactobacillus* isolates. Supernatants of pathogens were discarded since they were not injected in the larvae. Several dilutions of bacterial suspensions and washed cells were made using PBS, which were used for the injection experiment. On the day of injection, a plate count was carried out for all bacterial dilutions on culture agar media including: MRS and TSA for *Lactobacillus* and pathogens, respectively. The number of colony forming units (CFUs) per the individual larva was calculated in each bacterial solution which was injected in the larvae (Miles *et al.* 1938).

6.3.1.3 *Galleria mellonella* Treatments

In preliminary experiments, *Lactobacillus* injection was carried out using three groups of larvae, ten larvae were injected in each group. The first group was injected with 10^3 - 10^6 CFU/larvae of bacterial suspensions. The second group was injected with 10^1 - 10^7 CFU/larvae of bacterial washed cells, while the third group was injected with dilutions of filtered sterilised supernatants ranging from 10^0 - 10^{-3}. Regarding the pathogenic species, these were injected as bacterial suspensions and washed cells in several doses ranging from 10^1 - 10^6 CFU/larva. All the injected bacterial doses were monitored for their lethal potential on the larvae and survival percentages were reported during the incubation days of experiments. Furthermore, determination of the LD_{50} value for each pathogen was also performed, this value is the lethal dose of pathogenic bacterial species that kills half (50%) of the infected larvae at the end of the experiment duration.

6.3.1.4 Co-injection of Pathogens and *Lactobacillus* Species

Within the context of therapy, *G. mellonella* larvae were injected with *Lactobacillus* isolates as a means to treat skin infections causing pathogens. *Lb. delbreuckii* (Lb19) and *Lb. plantarum* (Lb21) isolated from yogurt and olives, respectively, were chosen to be injected separately with each of the three pathogens. Each of the two *Lactobacillus* species was injected in two different doses of bacterial washed cells. The selection of these two doses was done depending on the highest survival percentages obtained from the injection of *Lactobacillus* species in preliminary experiments (section 6.2.1.2). The doses were 10^2 and 10^4 CFU/larva for

Lb19, while they were 10^3 and 10^4 CFU/larva for Lb21. After the injection of several doses of pathogenic washed cells (10^1 - 10^6 CFU/larva), larvae were subsequently injected with each of the two selected doses of *Lactobacillus* species. Four control groups were included per experiment and each group had a total of ten larvae: the first group of larvae were injected with 10 μl of the pathogenic dose via the last left (L) proleg and 10 μl of PBS via the last right (R) proleg (e.g., SA1 10^6/L + PBS/R). The second group were injected with 10 μl of the first selected dose of *Lactobacillus* via the last left proleg and 10 μl of PBS via the last right proleg (e.g., Lb19 10^4/L + PBS/R). The third group were injected with 10 μl of the second selected dose of *Lactobacillus* via the last left proleg and 10 μl of PBS via the last right proleg (e.g., Lb19 10^2/L + PBS/R). The fourth group were injected with 10 μl of PBS via each of the two prolegs (e.g., PBS/L + PBS/R). The experiment was repeated twice for each pathogen. The number of survived larvae was recorded as percentages each day for six days post infection. For each co-injection experiment, comparisons between injected groups of larvae were performed. Statistical analysis was conducted for levels of compared groups and statistical differences were reported in tables related to the challenge experiment of each pathogen. Survival plot figures show *p*-values ≤ 0.05 of the compared groups which were significantly different. They also present *p*-values ranging from 0.06 - 0.09 that were considered to be trending towards significance. Non-significant differences with *p*-values > 0.05 were only reported in comparison tables, but not in the associated figures of survival plots.

6.3.1.5 Statistical Analysis

Survival percentages of all injection experiments were plotted throughout the incubation days. Statistical analysis was performed by using a non-parametric analysis of variance (one-way ANOVA) for multiple groups in RStudio software version 3.6.1. Dunn test Kruskal-Wallis test was carried out to compare the survival percentages obtained at the end of the incubation time. Comparisons were conducted between each injected group of larvae and the control group. Furthermore, for co-injection experiments, other comparisons were also done between two groups of injected larvae. Significant differences were reported at the threshold of *p*- value $\leq$ 0.05. The R script used for the statistical analysis is mentioned in Appendix 11.

6.3.2 Novel Infection Technique of *G. mellonella* Larvae (Topical Application)

The injection was performed in this study as a classical infection technique of larvae. In addition to this technique, a novel strategy was also carried out to infect the larvae with skin pathogenic species by the topical application of pathogens to the larvae body surface, followed by the application of *Lactobacillus* on top of the infected area. In this experiment, larvae were infected with *S. pyogenes* (SPA1) and treated with *Lb. plantarum* (Lb21). Ten larvae were used for each experimental group and control group. The larvae were placed in a Petri dish and each larva was separately fixed on a specific position of the plate by using an autoclave tape to prevent the larvae from movement during the bacterial inoculum application and to avoid the contact of the infected area by other larvae. Each larva was labelled on its dorsum by drawing two separate lines using a marker. The area between the two lines was sanitised with a sterile swab soaked in 70% ethanol. On the day of experiment, washed cells were prepared from the overnight culture of SPA1 as shown in section 6.2.1.2, and several decimal dilutions were made from the washed cells solution using 1x PBS (0.01 M) of PBS. The bacterial cells number was estimated in the washed cells original solution and in all of the prepared pathogenic doses by the performance of plate count on TSA medium. The number of CFUs per larva was calculated in each bacterial dose that was topically applied to the larvae.

Two preliminary trials were carried out to explore the appropriate technique for the topical infection of larvae. In the first trial, 10 μl inoculum of each pathogenic dose was directly transferred by a micropipette onto the intact sanitised area. Whereas the second trial of infection was performed by scratching the area with a sterile scalpel No10, followed by the pathogen inoculation at the same amount. After the application of the bacterial inoculum, the transferred amount was spread by the tip of the micropipette within the area determined by the two drawn lines. On the same day, washed cells and cell free supernatant (CFS) were prepared from the overnight culture of *Lb. plantarum* (Lb21) following the same steps mentioned in section 6.2.1.2. The plate count on MRS medium and the calculation of the CFUs number per larva were both conducted for the washed cells solution. Infected larvae were assigned into two groups, the first group was topically administered with 10 μl of *Lactobacillus* undiluted washed cells, while the second group was treated by the application of 10 μl of *Lactobacillus* undiluted supernatant. Both undiluted washed cells and CFS were applied over the infected area at 1 h post inoculation with each of the pathogenic doses. Four control groups of ten larvae were used in this

experiment. In the first group, larvae were topically administered with 10 μl of the pathogenic dose (four pathogenic doses). Both the second and third groups were applied with 10 μl of *Lactobacillus* undiluted washed cells and undiluted supernatant, respectively. In the fourth control group, 10 μl of PBS was applied to the dorsum of uninfected larvae. Larvae were incubated in the dark at 37ºC and the progress of injury was monitored during six days. For all treatments topically applied, the number of injured and dead larvae were counted and recorded daily until the last day of incubation. To assess the trauma severity, the count of larvae was carried out in groups scored from 0 - 3 which described the injury severity score (ISS). The injury scores were classified into: S0 = Absent (Not injured), S1 = Mild, S2 = Severe and S3 = Dead. Larvae were considered dead when they displayed no movement upon stimulation of touch with forceps.

6.4 Results

6.4.1 Injection of *Lactobacillus* Species into *G. mellonella*

6.4.1.1 Injection of *Lb. delbreuckii* Bacterial Isolates

Lactobacillus isolates were injected as three treatments including: bacterial suspensions, cell free supernatants and washed cells. For each treatment, Survival of the larvae was monitored on each day during the three-day incubation. Injection of *Lb. delbreuckii* isolates (Lb15, Lb17, Lb18 and Lb19) in the bacterial dose 10^6 CFU/larva, significantly affected the survival of larvae and showed either no larvae survived or low survival percentages. These percentages were between 0% - 40% for all injected bacterial suspensions (Figure 6.1A, B, C and D), and 0% - 50% for injected washed cells of the isolates Lb15, Lb17 and Lb18 (Figure 6.2A, B and C). However, 80% of larvae survived when the same dose 10^6 CFU/larva of Lb19 washed cells was injected, indicating that this isolate among all *Lb. delbreuckii* isolates was less virulent to the larvae when injected in this dose as washed cells (Figure 6.2D). To confirm these results, the injection of Lb19 washed cells was repeated in triplicate and the obtained survival percentages rate was 83.3%, which is close to the percentage of the preliminary trial. Statistical analysis was undertaken for the survival percentages of larvae injected with Lb19 washed cells. All larval groups included in each injected dose were compared with the control group (PBS) using non-parametric test Dunn Kruskal-Wallis test for multiple comparisons in R-Studio software (version 1.2.1335) and significant differences were calculated with a p-value ≤ 0.05. No significant

differences were detected among all groups, except the highest dose of bacterial cells 10^7 CFU/larva which was significantly different compared to the PBS with $p = 0.002$ (Figures 6.2D). Figure 6.4 showed larvae groups injected with different doses of *Lb. delbreuckii* washed cell after three days of incubation. Bacterial suspensions of *Lb. delbreuckii* isolates which were injected in a dose ranging between 10^3 - 10^5 CFU/larva, showed high survival percentages with a range of 80% - 100% on the third day of incubation (Figure 6.1A, B, C and D). Injection with the same range of doses 10^3 - 10^5 CFU/larva of bacterial washed cells displayed lower survival percentages ranging between 60% - 100% (Figure 6.2A, B, C and D).

Since larval survival was not highly affected by the injection of 10^2 CFU/larva and 10^4 CFU/larva of Lb19 washed cells, thus these two doses were selected for the challenge experiment (injection of pathogen and *Lactobacillus* washed cells). These two doses revealed high survival percentages 90% and 86.7%, respectively. Furthermore, they both showed no significant differences when compared to the PBS control group. Injection of undiluted cell free supernatants (CFSs) and 10^{-1} - 10^{-3} diluted supernatants of Lb15 and Lb17 isolates presented survival percentages ranging between 80% - 100%. While these percentages were between 20% - 80% for Lb18 and Lb19 supernatants (Figures 6.3A, B, C and D). For more confirmation, the injection of Lb19 supernatant was performed in triplicate and the highest survival percentage was 80% when the larvae injected with the dilution 10^{-1} of supernatant. Comparisons between each of supernatant groups and the PBS control group show significant differences with *p*-values ranging between 0.01 - 0.03, except the dilution 10^{-1} which presented no significant difference when compared with the PBS group (Figure 6.3D).

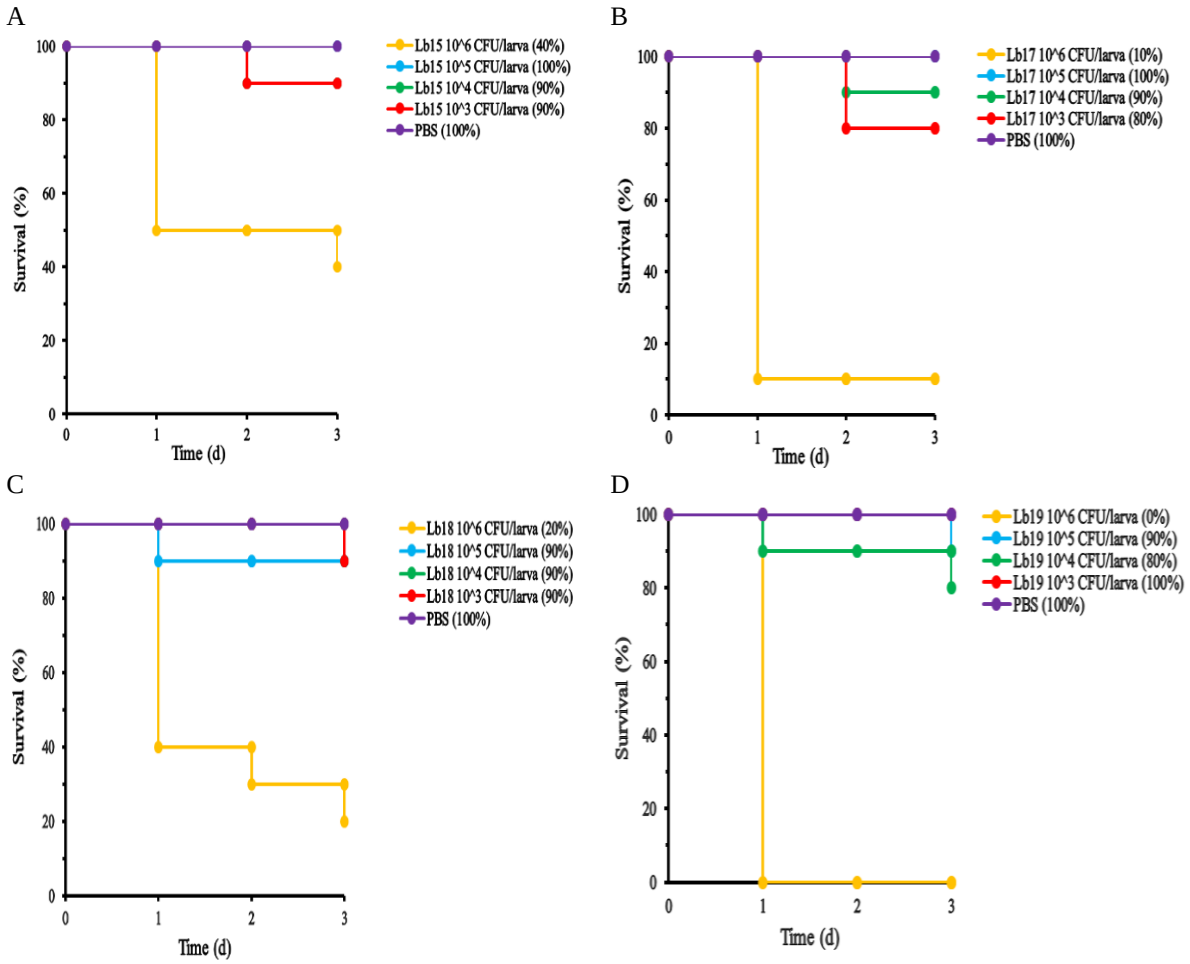


Figure 6.1 Survival plots of *G. mellonella* larvae injected with different doses of *Lb. delbreuckii* bacterial suspensions through three-day incubation. **A:** Lb15, **B:** Lb17, **C:** Lb18 and **D:** Lb19. Larval survival percentages of each group at the end of experiments are given between brackets in the legend. All experiments were performed once.

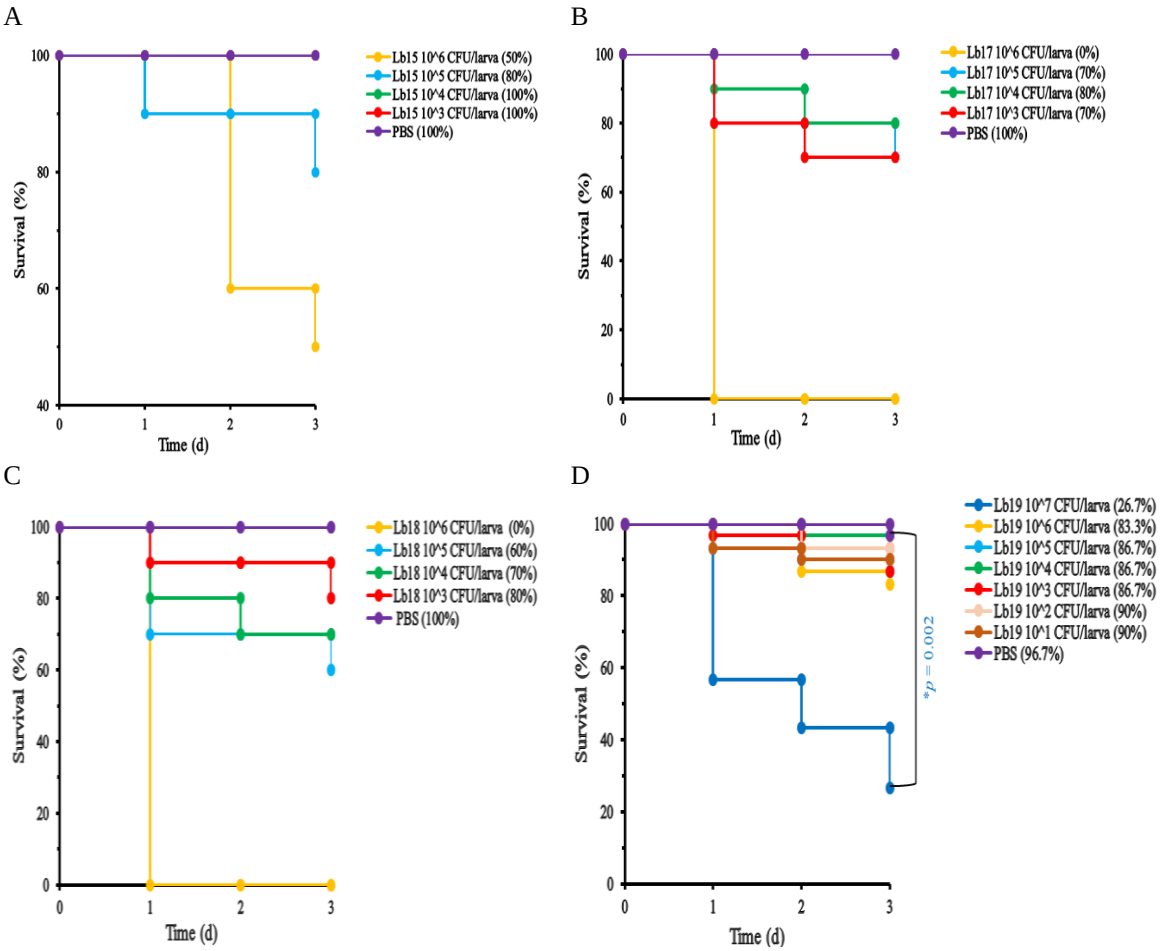


Figure 6.2 Survival plots of *G. mellonella* larvae injected with different doses of *Lb. delbreuckii* bacterial washed cells through three-day incubation. **A:** Lb15, **B:** Lb17, **C:** Lb18 and **D:** Lb19. Larval survival percentages of each group at the end of experiments are given between brackets in the legend. Experiments A, B and C were performed once while experiment D was conducted in triplicate. Each bacterial dose was compared with the PBS injected group (control) using non- parametric test Dunn Kruskal-Wallis test for multiple comparisons in R-Studio software. Black line shows significant differences with a p-value ≤ 0.05.

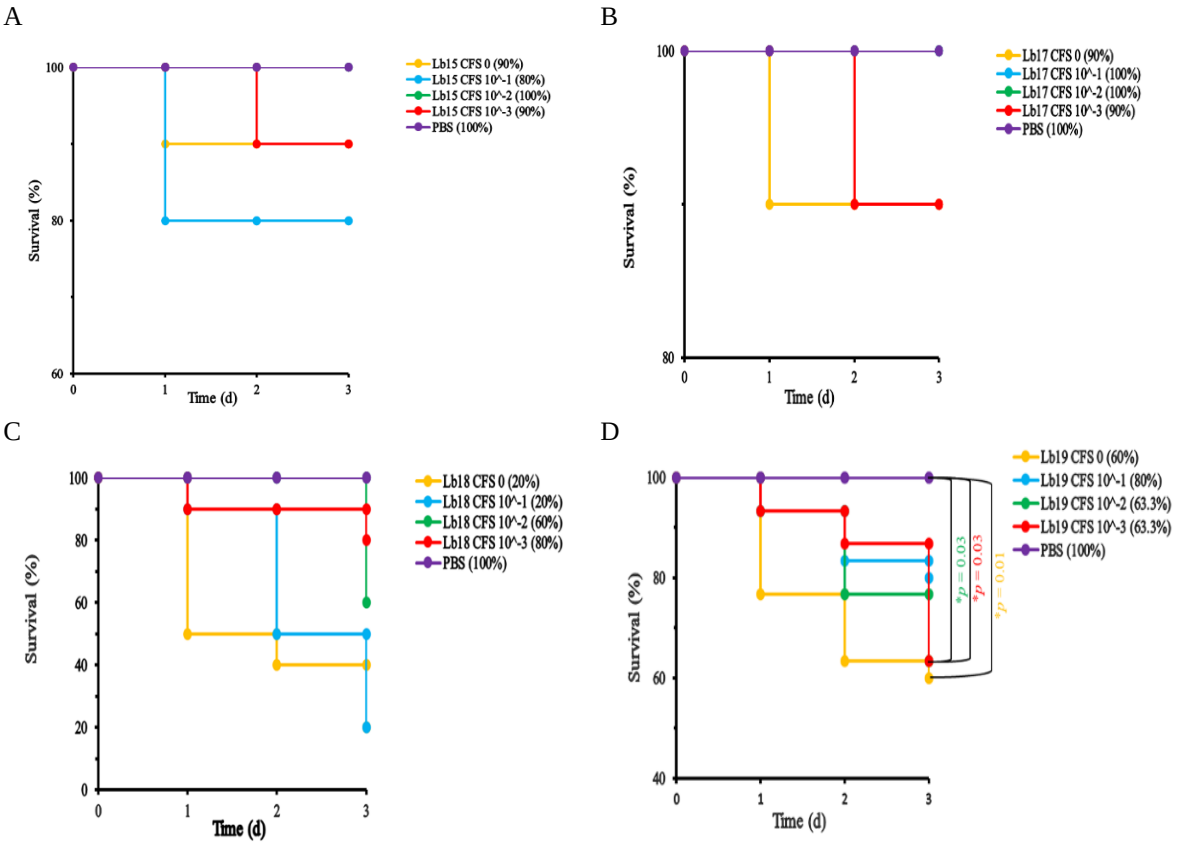


Figure 6.3 Survival plots of *G. mellonella* larvae injected with different dilutions of *Lb. delbreuckii* cell free supernatants (CFSs) through three-day incubation. **A:** Lb15, **B:** Lb17, **C:** Lb18 and **D:** Lb19. Larval survival percentages of each group at the end of experiments are given between brackets in the legend. Experiments A, B and C were performed once while experiment D was conducted in triplicate. Each dilution was compared with the PBS injected group (control) using non- parametric test Dunn Kruskal-Wallis test for multiple comparisons in R-Studio software. Black lines show significant differences with a p-value ≤ 0.05.

Figure 6.4 Survival of *G. mellonella* larvae injected with different doses (10^1-10^7 CFU/larva) of *Lb. delbreuckii* (Lb19) bacterial washed cells after three-day incubation. The upper left plate is the control group of larvae that received PBS.

6.4.1.2 Injection of *Lb. plantarum* Bacterial Isolates

Isolates of *Lb. plantarum* (Lb20-Lb25) injected at the dose of 10^6 CFU/larva as bacterial suspensions, presented no larvae survived or low survival percentages ranging between 0% - 20% after three-day incubation (Figure 6.5A, B, C, D, E and F). Similar survival percentages range was obtained when the same dose (10^6 CFU/larva) of washed cells was injected in the larvae (Figure 6.6A, B, C, D, E and F). This indicates that this dose had a high lethal effect on the survival of larvae. Larval groups receiving 10^5 CFU/larva of bacterial suspensions showed different survival percentages for the six isolates. These percentages were high ranging between 60% - 100% for Lb21, Lb23, Lb24 and Lb25, while they were low 10% and 20% for Lb20 and Lb22, respectively (Figure 6.5A, B, C, D, E and F). Different survival percentages also obtained when the same dose 10^5 CFU/larva was injected as washed cells. Survival percentages range was 60% - 80% for the four isolates Lb22 - Lb25, whereas only 30% and 33.3% of injected larvae survived for Lb20 and Lb21, respectively (Figure 6.6A, B, C, D, E and F).

Regarding the injection of the doses 10^3 CFU/larva and 10^4 CFU/larva of bacterial suspensions, the obtained survival percentages were high ranging between 70% - 100% for all isolates except Lb20 that revealed low survival percentages when these two doses were injected (Figure 6.5A, B, C, D, E and F). High survival percentages were also found when the same two doses 10^3 CFU/larva and 10^4 CFU/larva were injected as washed cells, these percentages were

between 80% - 100% for all isolates with an exception of Lb20 at the dose 10^4 CFU/larva which showed only 50% of larvae survived (Figure 6.6A, B, C, D, E and F).

The injection of washed cells was conducted in triplicate for Lb21, the results showed high survival percentages approaching more than 80% when the doses range 10^1 - 10^4 CFU/larvae was injected. Comparisons of high doses with PBS control group indicated statistically significant differences with *p* values of 0.01 and 0.001 for 10^5 CFU/larva and 10^6 CFU/larvae, respectively. No significant differences were shown when survival percentages of lower doses were compared to those of the control group. Thus, both of 10^3 CFU/larva and 10^4 CFU/larva were nominated for injection with pathogens in challenge experiments. Moreover, these two doses presented high survival percentages which were 90% for the first dose and 83.3% for the second one (Figures 6.6B and 6.8).

It was observed that the injection of undiluted cell free supernatants exhibited low percentages of larvae survived. Most of these percentages gradually increased to a maximum of 100% when diluted CFSs were injected (Figure 6.7A, B, C, D, E and F). In terms of Lb21 supernatant, this was injected in triplicate for more confirmation. A low survival percentage of 26.7% was obtained when the undiluted supernatant was injected. Moreover, injection of diluted supernatants 10^{-1} and 10^{-2} also presented low survival percentages equal or less than 50%. Therefore, the undiluted and the first two diluted supernatants were all significantly different to PBS group with *p*-values <0.05. Since the injection of 10^{-3} diluted supernatant showed a survival percentage of 70%, no significant difference was found between this survival percentage and that of the PBS injected group (Figure 6.7B).

Table 6.1 illustrates the highest survival percentages of *G. mellonella* injected with *Lb delbreuckii* and *Lb. plantarum* bacterial suspensions, washed cells and supernatants. Injected bacterial doses or supernatant dilutions mentioned in the table are those that showed the highest percentages of larvae survived after three-day incubation.

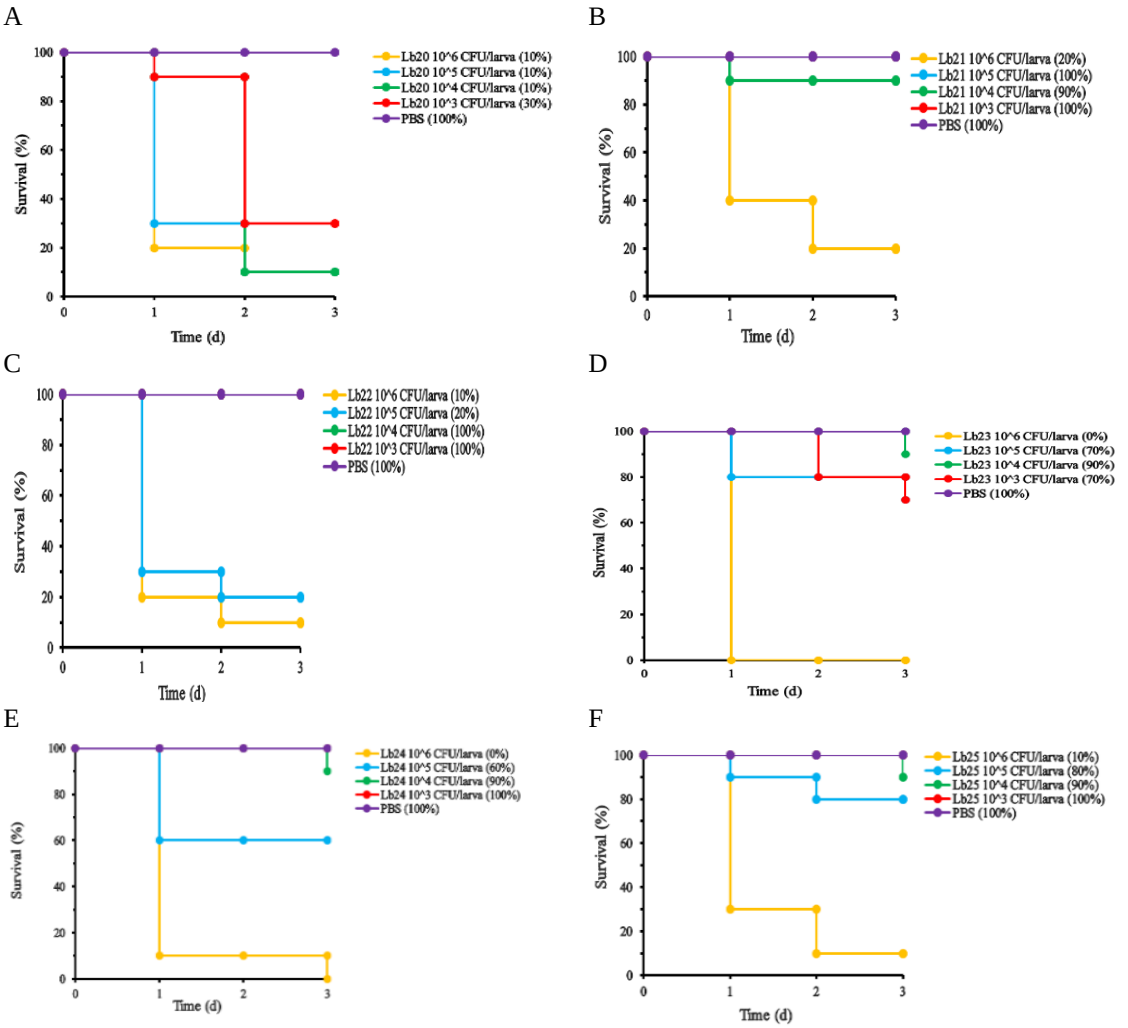


Figure 6.5 Survival plots of *G. mellonella* larvae injected with different doses of *Lb. plantarum* bacterial suspensions isolated from olives through three-day incubation. **A:** Lb20, **B:** Lb21, **C:** Lb22, **D:** Lb23, **E:** Lb24 and **F:** Lb25. Larval survival percentages of each group at the end of experiments are given between brackets in the legend. All experiments were performed once.

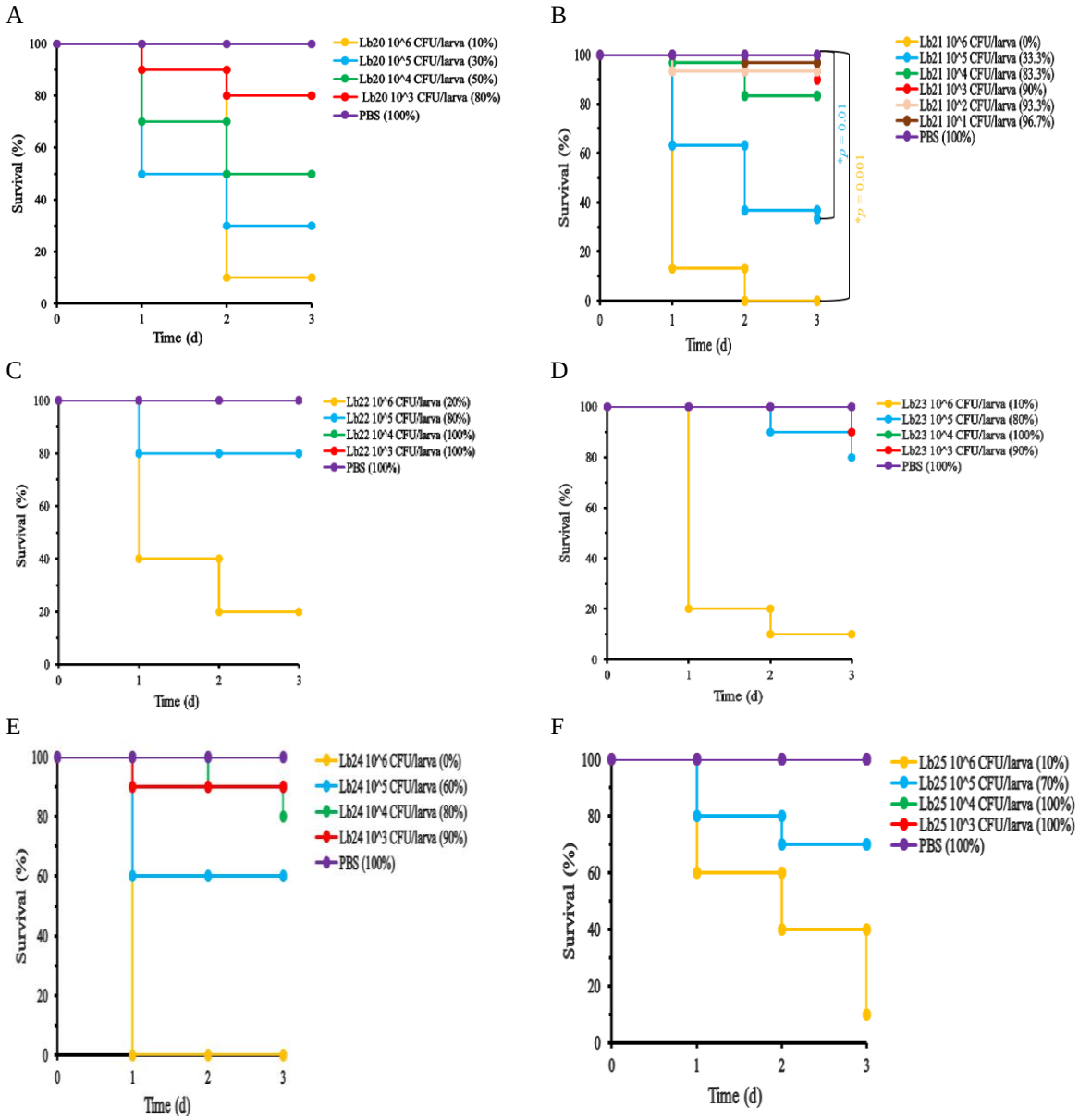


Figure 6.6 Survival plots of *G. mellonella* larvae injected with different doses of *Lb. plantarum* bacterial washed cells through three-day incubation. **A:** Lb20, **B:** Lb21, **C:** Lb22, **D:** Lb23, **E:** Lb24 and **F:** Lb25. Larval survival percentages of each group at the end of experiments are given between brackets in the legend. Experiments A, C, D, E and F were performed once while B was done in triplicate. Each bacterial dose was compared with the PBS injected group (control) using non- parametric test Dunn Kruskal-Wallis test for multiple comparisons in R-Studio software. Black lines show the significant differences with a p-value $\leq$ 0.05.

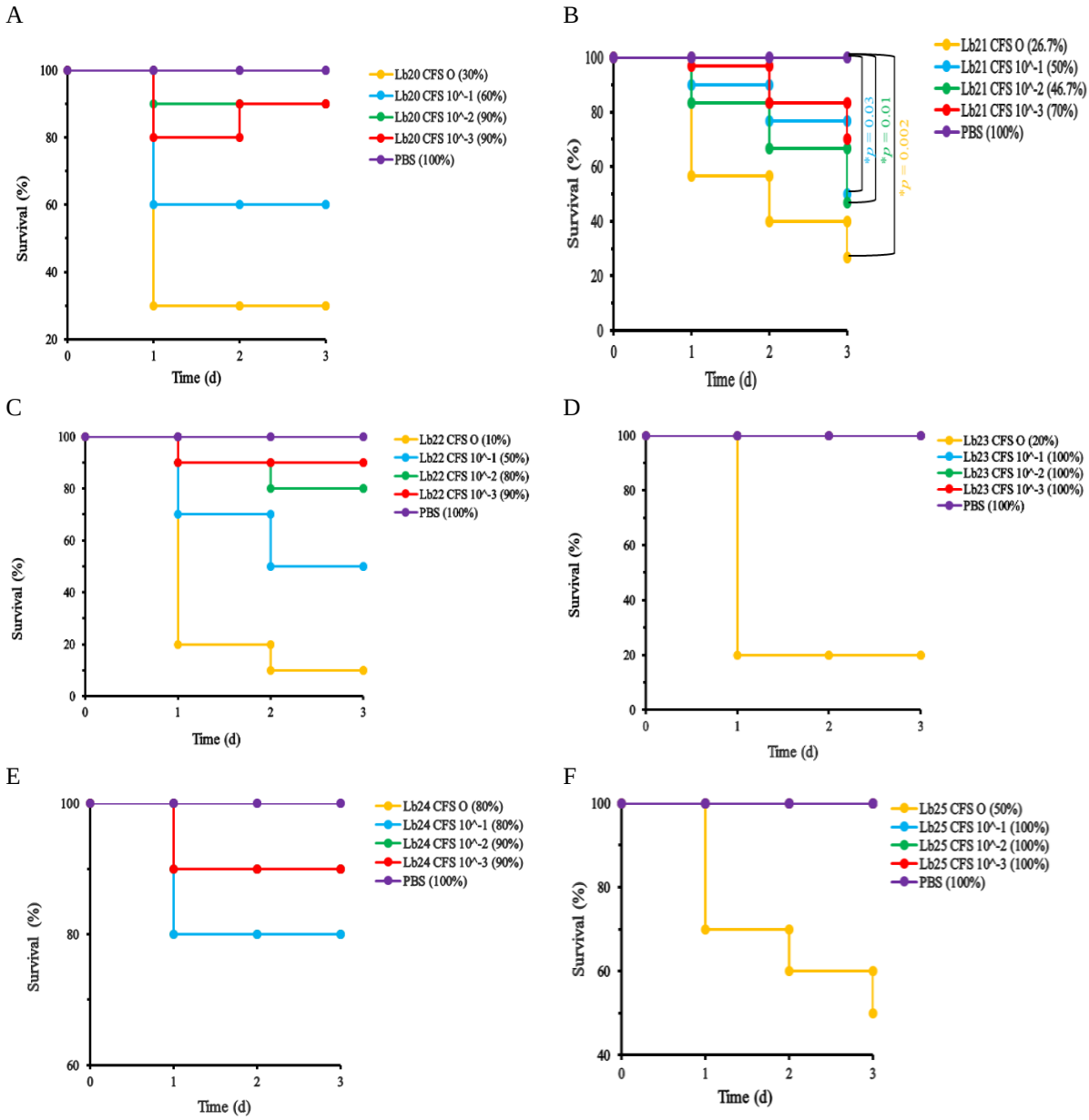


Figure 6.7 Survival plots of *G. mellonella* larvae injected with different dilutions of *Lb. plantarum* cell free supernatants through three-day incubation. **A:** Lb20, **B:** Lb21, **C:** Lb22, **D:** Lb23, **E:** Lb24 and **F:** Lb25. Larval survival percentages of each group at the end of experiments are given between brackets in the legend. A, C, D, E and F were performed once while B was done in triplicate. Each dilution was compared with the PBS injected group (control) using non- parametric test Dunn Kruskal-Wallis test for multiple comparisons in R-Studio software. Black lines show the significant differences with a p-value ≤ 0.05.

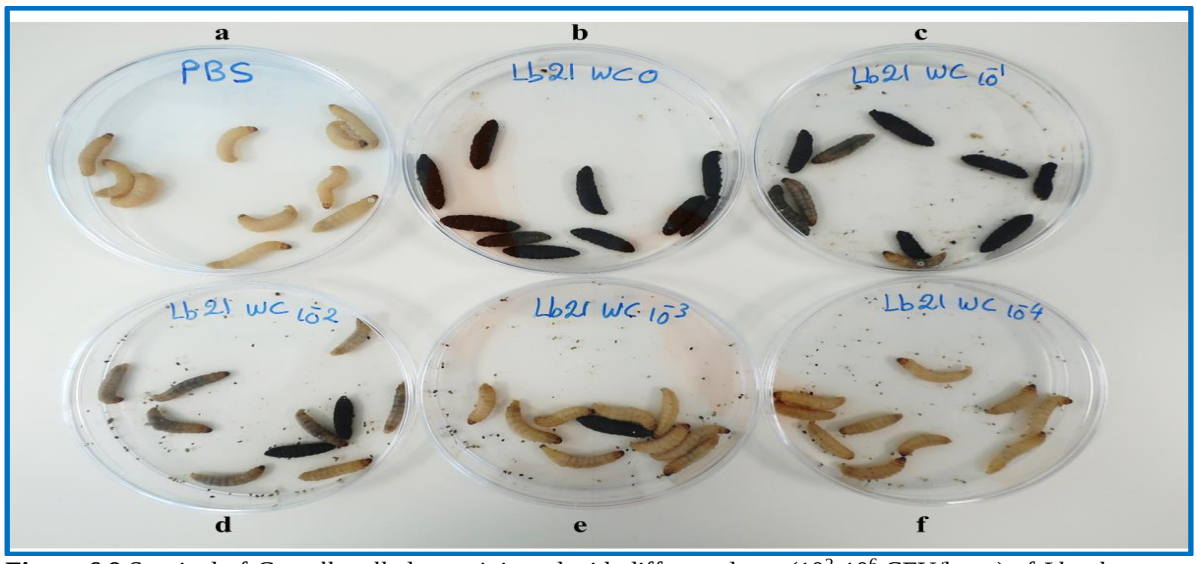


Figure 6.8 Survival of *G. mellonella* larvae injected with different doses (10^2-10^6 CFU/larva) of *Lb. plantarum* (Lb21) bacterial washed cells after three-day incubation. a: PBS (control), b: 10^6, c: 10^5, d: 10^4, e: 10^3 and f: 10^2.

Table 6.1 The highest survival percentages of *G. mellonella* larvae injected with specific doses of *Lb delbreuckii* (Lb15, Lb17, Lb18 and Lb19) and *Lb. plantarum* (Lb20, Lb21, Lb22, Lb23, Lb24 and Lb25) bacterial suspensions, washed cells and supernatants after three-day incubation

Lb Isolate	Bacterial Dose or Solution (The Highest Survival Percentages %)		
	Bacterial Suspensions[1]	Bacterial Washed Cells[1]	Bacterial Supernatants[2]
Lb15	10^5 (100)	10^3-10^4 (100)	10^{-2} (100)
Lb17	10^5 (100)	10^4 (80)	10^{-1}-10^{-2} (100)
Lb18	10^3-10^5 (90)	10^3 (80)	10^{-3} (80)
Lb19	10^3 (100)	10^1-10^2 (90) (Triplicate)	10^{-1} (80) (Triplicate)
Lb20	10^3 (30)	10^3 (80)	10^{-2} (90)
Lb21	10^3, 10^5 (100)	10^1 (96.7) (Triplicate)	10^{-3} (70) (Triplicate)
Lb22	10^3-10^4 (100)	10^3-10^4 (100)	10^{-3} (90)
Lb23	10^4 (90)	10^4 (100)	10^{-1}-10^{-3} (100)
Lb24	10^3 (100)	10^3 (90)	10^{-1} (80)
Lb25	10^3 (100)	10^3-10^4 (100)	10^{-1}-10^{-3} (100)

[1]Both of the bacterial suspensions and washed cells doses are presented in CFU/larva.
[2]Bacterial supernatants are shown in serial dilutions of cell free supernatants (CFSs).

6.4.2 Injection of Pathogenic Species into *G. mellonella*

6.4.2.1 Injection of *S. aureus* (SA1)

Injection of larvae with pathogenic bacteria was carried out in triplicate as bacterial suspensions and washed cells. Control groups of larvae injected with PBS showed survival percentages of 96.7% for bacterial suspensions and 100% for washed cells after three-day incubation. Injection with 10^6 CFU/larva of either bacterial suspension or washed cells of *S. aureus* revealed rapid killing of larvae which was noticed after the first day of infection. The survival rate of larvae infected with this dose approached 6.7% for the bacterial suspension after three-day incubation (Figure 6.9 A and B), while this percentage was 0% for the washed cells on the second day of incubation (Figure 6.9 C and D). Comparison of these survival percentages with the PBS control group showed high significant differences with *p*-values 0.004 for the bacterial suspension and 0.001 for washed cells. When both of bacterial suspensions and washed cells have been diluted, the survival percentages gradually increased. The significant difference between 10^5 CFU/larva and the control group was lower than the previous bacterial dose with a *p*-value 0.01 for both of the washed cells and suspensions. Determination of LD_{50} values showed that this value was 10^4 CFU/larva for bacterial suspensions, once the survival percentage approached 33.3% after three days. The statistical difference was trending to significance with a *p*-value 0.06 when this percentage was compared to the PBS control group. Regarding the pathogenic washed cells, injection of both 10^3 CFU/larva and 10^4 CFU/larva presented 50% of larvae survived after three-day incubation. Thus, these two doses were designated as LD_{50} values and their comparison with the control group showed significant differences with a *p*-value of 0.05 for both doses. Lower bacterial doses reveal no significance difference in comparison with the larval group injected with PBS.

6.4.2.2 Injection of *S. pyogenes* (SPA1)

In terms of *S. pyogenes*, injection of the bacterial suspension at a dose of 10^5 CFU/larva (the highest injected dose) presented a survival percentage of 3.3% after 72 h. When this dose was compared to the larval survival of the control group (96.7%), it was highly significant different with a *p*-value 0.004. Survival percentages started to increase by injection of diluted suspensions that have lower number of bacterial cells than the original bacterial suspension. After three-day incubation, LD_{50} dose was 10^3 CFU/larva with a survival percentage of 43.3%. A sudden decline in survival rates to 10% - 13.3% was observed on the third day after injection

of 10^1 - 10^2 CFU/larva of bacterial suspensions (Figure 6.10 A), which means that low doses of bacterial suspension were also lethal. To investigate the reason of this virulence, culture medium (TSA) (which used for pathogens cultivation) was examined for its toxic effect on the survival of larvae. This was performed by injection of the uninoculated medium inside the larvae which resulted in high survival percentages (90%, data not shown).

Several doses of *S. pyogenes* washed cells ranging between 10^1 - 10^6 CFU/larva were also injected inside the larvae. Groups of larvae that received 10^6 CFU/larva all died after the first day of infection resulting in a survival rate of 0%. Since all the other injected bacterial doses (10^1 - 10^5 CFU/larva) revealed survival percentages higher than 50% after three days of infection, the LD_{50} dose could not be estimated during this time of incubation. Therefore, the incubation period was extended to six days to check the survival of larvae and find out the LD_{50} dose. On the sixth day of incubation, the LD_{50} dose was determined as 10^5 CFU/larva with a survival rate of 33.3%. As larval survival percentage of the PBS control group approached 46.7% at the end time point of incubation, no significant differences were observed between the PBS group and any of the injected doses of bacterial washed cells (Figures 6.10 B and C).

6.4.2.3 Injection of *S. dysgalactiae* subsp *equisimilis* (SDG4)

G. mellonella larvae were entirely killed by injection of 10^6 CFU/larva of *S. dysgalactiae* subsp *equisimilis* (SDG4) bacterial suspension after the first day of injection. Therefore, this bacterial dose was statistically different to the control group with a *p*-value of 0.001. Dilution of the bacterial suspension showed an increase in survival rates to 73.3% after 72 h of injecting the suspension containing 10^1 CFU/larva. The dose that has approximately killed half of the larvae (46.7%) at the end of the incubation's period was 10^3 CFU/larva which presented no significant difference to the larvae receiving PBS (Figure 6.11 A and B). Since larvae survival percentages remained high after three days of infection with bacterial washed cells, the incubation period was extended for six days to obtain the LD_{50} dose. After six days of infection, the survival percentage approached 60% when 10^5 CFU/larva of washed cells was injected inside the larvae and was significantly different to the control group with a *p*-value of 0.004. Whereas injection of 10^1 - 10^4 CFU/larva showed high survival rates (90% - 100%) despite of the expanded incubation time. Thus, it was not possible to obtain the LD_{50} dose of the bacterial washed cells (Figure 6.11 C and D).

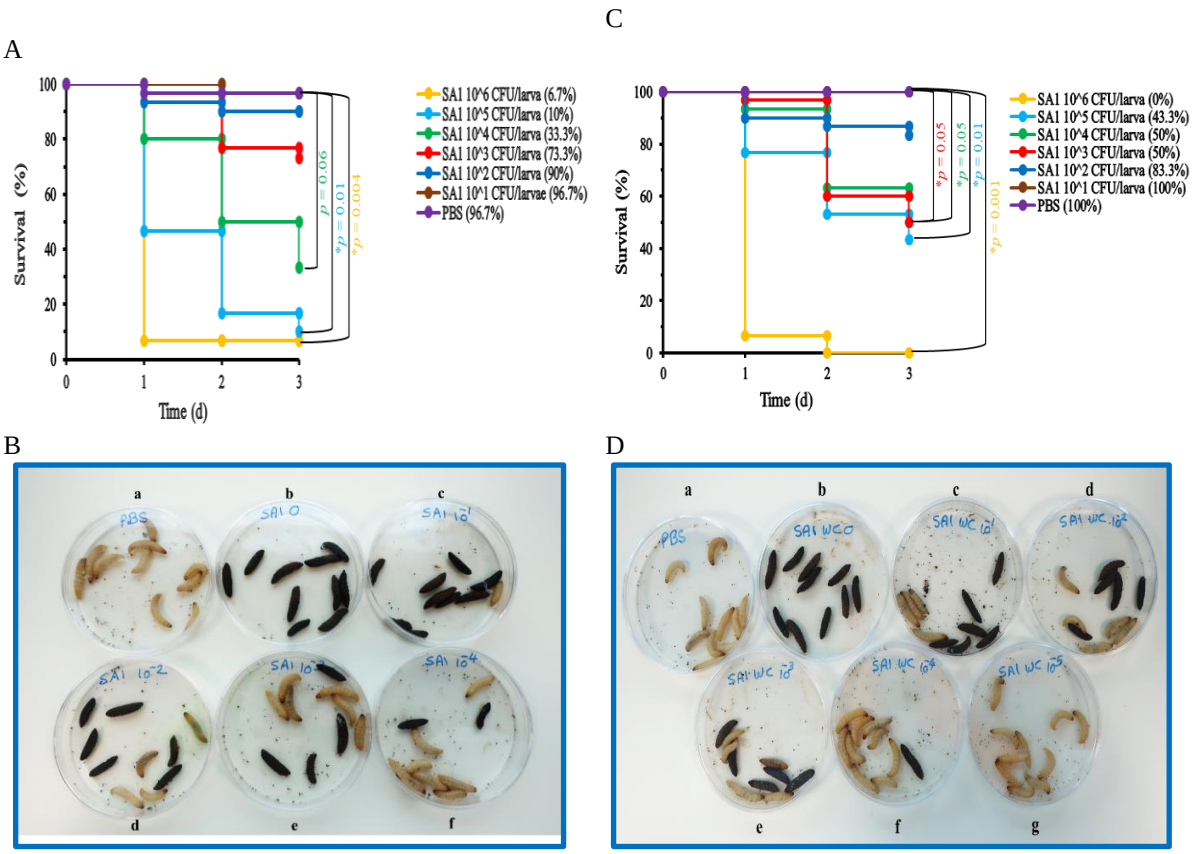
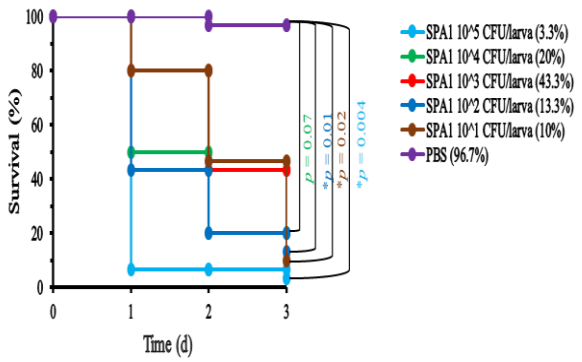


Figure 6.9 Survival of *G. mellonella* larvae injected with different doses (10^1-10^6 CFU/larva) of *S. aureus* bacterial suspension and washed cells through three-day incubation. **A** and **C**: survival plots, larval survival percentages at the end of experiments are given between brackets in the legends. Experiments were conducted in triplicate; each bacterial dose was compared with the PBS injected group (control) using non- parametric test Dunn Kruskal-Wallis test for multiple comparisons in R-Studio software. Black lines show significant differences with a *p*-value ≤ 0.05. **B** and **D**: injected groups of larvae after three-day incubation. **A**: survival plot of the larvae injected with bacterial suspension, LD_{50} (33.3%) was obtained after three-day incubation of larvae injected with 10^4 CFU/larva. **B**: Groups of larvae injected with bacterial suspension. a: PBS (control), b: 10^6, c: 10^5, d: 10^4 (LD_{50}), e: 10^3 (LD_{50}) and f: 10^2. **C**: survival plot of the larvae injected with washed cells, LD_{50} (50%) was obtained after three-day incubation of larvae injected with 10^3-10^4 CFU/larva. **D**: Groups of larvae injected with washed cells. a: PBS (control), b: 10^6, c: 10^5, d: 10^4 (LD_{50}), e: 10^3 (LD_{50}), f: 10^2 and g: 10^1 CFU/larva.

A



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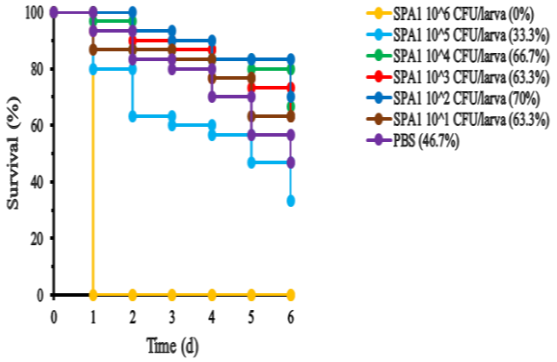

C

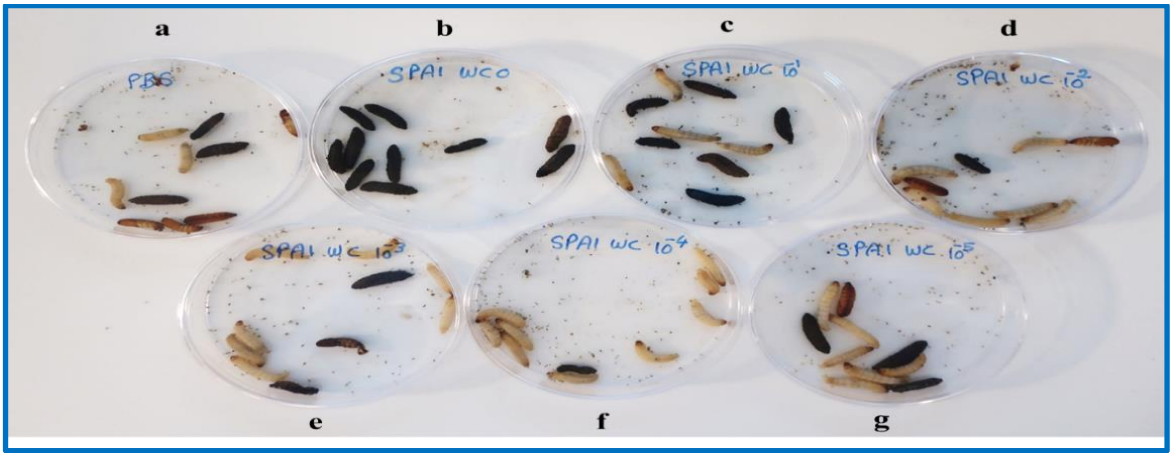

Figure 6.10 Survival of *G. mellonella* larvae injected with different doses of *S. pyogenes* bacterial suspension and washed cells through the incubation days. **A** and **B**: survival plots, larval survival percentages at the end of experiments are given between brackets in the legends. Experiments were conducted in triplicate; each bacterial dose was compared with the PBS injected group (control) using non- parametric test Dunn Kruskal-Wallis test for multiple comparisons in R-Studio software. Black lines show significant differences with a p-value ≤ 0.05. **A**: survival plot of the larvae injected with bacterial suspension (10^1-10^5 CFU/larva), LD_{50} (43.3%) was obtained after three-day incubation of larvae injected with 10^3 CFU/larva. **B**: survival plot of the larvae injected with washed cells (10^1-10^6 CFU/larva), LD_{50} (33.3%) was obtained after six days of incubation of larvae injected with 10^5 CFU/larva. **C**: Groups of larvae injected with washed cells after six days of incubation. a: PBS (control), b: 10^6, c: 10^5 (LD_{50}/33.3%), d: 10^4, e: 10^3, f: 10^2 and g: 10^1 CFU/larva.

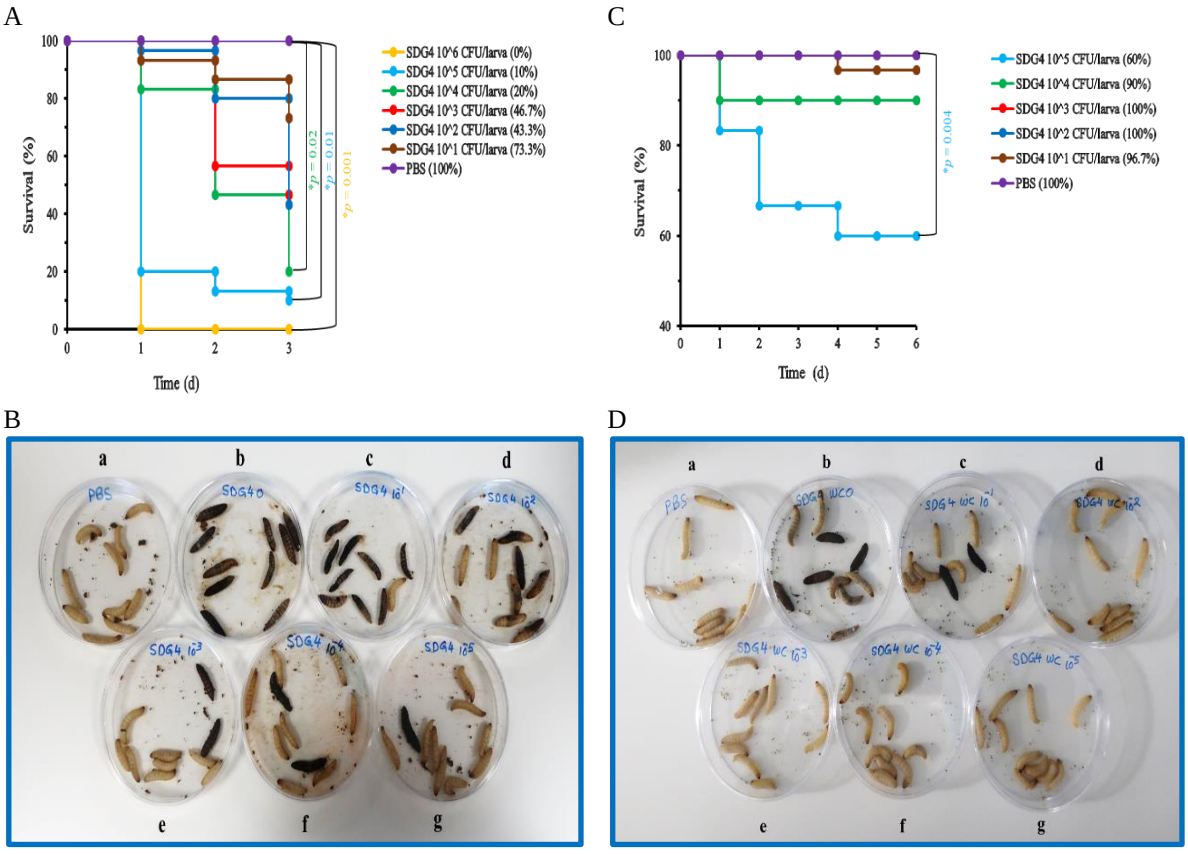

Figure 6.11 Survival of *G. mellonella* larvae injected with different doses of *Strep. dysgalactiae* subsp *equisimilis* (SDG4) bacterial suspension and washed cells through the incubation days. **A** and **C**: survival plots, larval survival percentages at the end of experiments are given between brackets in the legends. Experiments were conducted in triplicate; each bacterial dose was compared with the PBS injected group (control) using non- parametric test Dunn Kruskal-Wallis test for multiple comparisons in R-Studio software. Black lines show significant differences with a *p*-value ≤ 0.05. **B** and **D**: injected groups of larvae at the end of the incubation time. **A**: survival plot of the larvae injected with bacterial suspension (10^1-10^6 CFU/larva), LD_{50} (46.7%) was obtained after three-day incubation of larvae injected with 10^3 CFU/larva. **B**: Groups of larvae injected with bacterial suspension after three-day incubation. a: PBS (control), b: 10^6, c: 10^5, d: 10^4, e: 10^3 (LD_{50}), f: 10^2 and g: 10^1 CFU/larva. **C**: survival plot of the larvae injected with washed cells (10^1-10^5 CFU/larva), LD_{50} could not be obtained after six days' incubation. **D**: Groups of larvae injected with washed cells after six days of incubation. a: PBS (control), b: 10^5, c: 10^4, d: 10^3, e: 10^2 (LD_{50}), f: 10^1 and g: 10^0 CFU/larva (This group was injected with a solution that had no bacterial cells as the result of the CFU count was obtained the next day of the injection).

6.4.3 Co-injection of Pathogens and *Lactobacillus* Species into *G. mellonella*

6.4.3.1 Co-injection of *S. aureus* (SA1) and *Lb. delbreuckii* (Lb19)

The survival plots of *G. mellonella* injected with washed cells of *Lb. delbreuckii* (Lb19) as means to control *S. aureus* (SA1) infection are shown in Figure 6.12. Each dose of pathogenic bacterial cells, ranging from 10^1 - 10^6 CFU/larva, was individually plotted with two doses of Lb19 (10^2 CFU/larva and 10^4 CFU/larva) and four control groups (mentioned in section 6.2.1.3) as well. Table 6.2 depicts the comparison of groups of SA1 co-injected with Lb19 washed cells and statistical differences (*p*-values) of these compared groups.

Comparison of survival percentages (X%) between the group of larvae injected with a specific dose of SA1 and the control group receiving PBS, showed that pathogenic doses 10^6 CFU/larva (0%), 10^5 CFU/larva (20%), 10^4 CFU/larva (20%) and 10^3 CFU/larva (40%) were all significantly different to PBS group (95%) with *p*-values of 0.05 for the first two doses and 0.02 for the second two doses. While injection of 10^2 CFU/larva tended to trend to significance with higher survival percentage (65%) and *p*-value 0.06. No significant difference was indicated by injection of the lowest pathogenic dose 10^1 CFU/larva that resulted in the highest survival percentage (90%). As the doses range 10^3 - 10^6 CFU/larva revealed low survival percentages and significant differences (*p*-values ≤ 0.05) compared to PBS group, it was found that SA1 had a virulent effect on the larvae when it was injected at this range of doses.

Each pathogenic dose was also compared to the two Lb19 doses which both presented 90% of larvae survived. Comparison between 10^6 CFU/larva (0%) of pathogenic cells and each dose of Lb19 (90%), showed statistical differences that were trending towards significance with a *p-value* of 0.09. Similar result was obtained when the comparison was performed for SA1 10^5 CFU/larva (20%) with a *p*-value of 0.08. Further comparisons with 10^4 CFU/larva (20%) and 10^3 CFU/larva (40%) pathogenic cells resulted in significant differences with *p*-values of 0.03 and 0.04, respectively. While, no significant differences were found when both doses of Lb19 were compared to the lower injected doses of pathogenic washed cells 10^2 CFU/larva (65%) and 10^1 CFU/larva (90%). A large difference in survival percentages was observed between the high percentage (90%) obtained from the Lb19 doses injection, and low survival percentages (0% - 65%) resulted from the injection of 10^2 - 10^6 CFU/larva of SA1. This indicates that these doses of SA1 were more lethal than the injected Lb19 doses. Despite of this difference in survival percentages (25% - 90%), the statistical analysis showed significant differences for some

pathogenic doses but it was trending to significance for others. Similar lethal effect was shown by 10^1 CFU/larva of SA1 and both of Lb19 doses as they all had the same survival percentage (90%).

Groups of larvae injected with the pathogen and challenged with either 10^4 CFU/larva or 10^2 CFU/larva of Lb19 washed cells, were also compared to the PBS control group. The bacterial dose 10^6 CFU/larva of SA1 injected with either of the two Lb19 doses, revealed no larvae survived (0%). These groups were compared to the PBS group (95%) and the comparison resulted in a significance with a *p*-value of 0.05. Survival percentage of the larvae which received 10^5 CFU/larva of SA1 and 10^4 CFU/larva of Lb19 was 10%, this group was significantly different to the PBS with a *p*-value of 0.02. Although the larvae injected with the same pathogenic dose 10^5 CFU/larva and 10^2 CFU/larva of Lb19 showed a survival of 35%, the comparison with the PBS group was not significant with a *p*-value of 0.21. Larvae injected with any of the low pathogenic doses (10^1-10^4 CFU/larva) and Lb19 doses revealed different survival percentages (45% - 90%), but no significant differences were detected when those compared to the control group injected with PBS. Regardless of the absence of significance in the statistical analysis, any co-injected dose which revealed less than 50% of larvae survived and had a difference in survival percentage equal or more than 50% compared to PBS group, was recognised as the dose with a high lethal potential. Thus, co-injection doses which contained 10^4 - 10^6 CFU/larva of SA1 and any of Lb19 doses were highly lethal, while other co-injected doses had a less lethal effect on the larvae.

Another comparison was also carried out between two groups: the first group was the larvae co-injected with the pathogen and one of the two doses of Lb19, for example SA1 10^6 CFU/larva + Lb19 10^4 CFU/larva or 10^2 CFU/larva. The second group was the larvae receiving the same dose of Lb19 alone (Lb19 10^4 CFU/larva or 10^2 CFU/larva). Larvae injected with 10^6 CFU/larva of SA1 and any of Lb19 doses died (0%). This group was compared to the larvae injected with the same dose of Lb19 alone (90%), and the statistical analysis was trending to significance with a *p*-value of 0.09. While a significant difference with a *p*-value of 0.04 was observed when the larvae co-injected with SA1 10^5 CFU/larva and Lb19 10^4 CFU/larva (10%) was compared to the larvae injected with that dose of Lb19 alone (90%). Despite of the obvious difference between survival percentages of the group infected with SA1 10^5 CFU/larva and treated with Lb19 10^2 CFU/larva (35%) and the group receiving Lb19 10^2 CFU/larva itself (90%), no significant difference was obtained from the comparison of these two groups and the *p*-value was > 0.05. Survival percentages of larvae co-injected with low pathogenic doses (10^1 -

10^4 CFU/larva) and any of the two Lb19 doses, were compared to percentages of the corresponding injected Lb19 dose. The difference between survival percentages of these compared groups was small, thus no statistically significant differences were observed. Co-injected doses that contained low pathogenic cells (10^1 - 10^3 CFU/larva) and any of Lb19 doses, were considered less lethal doses, since they all revealed more than 50% of larvae survived and slight differences (0% - 35%) in survival percentages compared to the group injected with Lb19 doses alone.

To explore the therapeutic effect of Lb19 against SA1 infection, the survival percentage of each group infected with a pathogenic dose and either of the two doses of Lb19 was compared to the survival obtained from the injection of that pathogenic dose alone. Injection of the highest pathogenic dose 10^6 CFU/larva revealed a total death of larvae (0%), when it was injected alone or co-injected with any of the two doses of Lb19. Once the number of injected pathogenic cells decreased, an increase in the survival of larvae was observed. Injection of SA1 in a dose of 10^5 CFU/larva showed a survival of 20%, whilst the survival increased up to 90% once the larvae were infected with 10^1 CFU/larva of SA1. Groups of larvae co-injected with 10^2 - 10^4 CFU/larva of the pathogen and 10^4 CFU/larva of Lb19, showed an increase in survival of larvae ranging from 15% - 25% compared to the groups injected with the same pathogenic doses alone. However, no significant differences were obtained from statistical analysis performed for these groups. Regarding the bacterial dose 10^2 CFU/larva of Lb19, it was detected that the lethal effect of the pathogen declined for groups of larvae infected with higher pathogenic dose (10^2 - 10^5 CFU/larva) and injected with 10^2 CFU/larva of Lb19. Although this dose of Lb19 was more efficient to reduce the pathogen's virulence than 10^4 CFU/larva of Lb19, both doses had a therapeutic potential against the pathogenic infection. Moreover, the co-injection of both doses resulted in similar increase range of survival percentages which was 15% - 25% (Table 6.3). Figure 6.13 displays the therapeutic activity of both Lb19 doses against the infection of *S. aureus* which was injected in several doses.

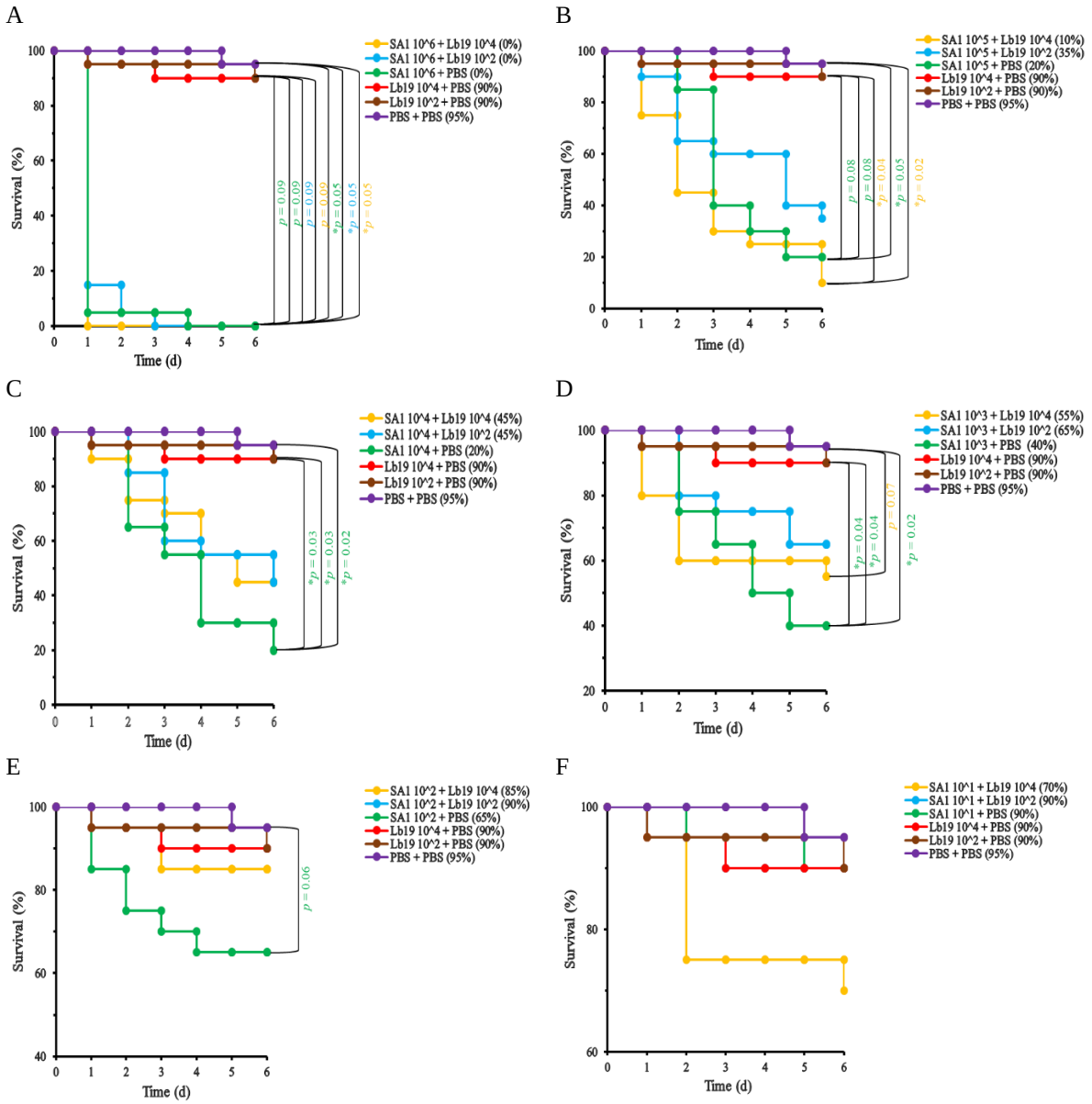

Figure 6.12 Survival plots of *G. mellonella* larvae injected with different doses (10^1-10^6 CFU/larva) of *S. aureus* (SA1) bacterial washed cells and treated with two doses of *Lb. delbreuckii* (Lb19) washed cells (10^4 and 10^2 CFU/larva) through six-day incubation. Larval survival percentages at the end of experiments are given between brackets in the legends. Experiments were conducted in duplicate; and comparisons among groups were performed using non- parametric test Dunn Kruskal-Wallis test for multiple comparisons in R-Studio software. Black lines show significant differences with a *p*-value ≤ 0.05. **A-F:** SA1 washed cells doses. **A:** 10^6, **B:** 10^5, **C:** 10^4, **D:** 10^3, **E:** 10^2 and **F:** 10^1 CFU/larva.

Table 6.2 Significant differences as determined by non- parametric test Dunn Kruskal-Wallis test for multiple comparisons for the co-injection of *S. aureus* (SA1) and *Lb. delbreuckii* (Lb19) at the sixth day post-infection.

Pathogen's dose (CFU/larva)	Levels of compared groups SA1 X Lb19 (10^4 and 10^2)				*p*-value
	Left Proleg	Right Proleg	Left Proleg	Right Proleg	
SA1 10^6	10^6	PBS	PBS	PBS	*0.05
	10^6	10^4	PBS	PBS	*0.05
	10^6	10^2	PBS	PBS	*0.05
	10^6	PBS	10^4	PBS	0.09
	10^6	PBS	10^2	PBS	0.09
	10^6	10^4	10^4	PBS	0.09
	10^6	10^2	10^2	PBS	0.09
	10^6	10^4	10^6	PBS	1.00
	10^6	10^2	10^6	PBS	1.00
SA1 10^5	10^5	PBS	PBS	PBS	*0.05
	10^5	10^4	PBS	PBS	*0.02
	10^5	10^2	PBS	PBS	0.21
	10^5	PBS	10^4	PBS	0.08
	10^5	PBS	10^2	PBS	0.08
	10^5	10^4	10^4	PBS	*0.04
	10^5	10^2	10^2	PBS	0.29
	10^5	10^4	10^5	PBS	0.78
	10^5	10^2	10^5	PBS	0.48
SA1 10^4	10^4	PBS	PBS	PBS	*0.02
	10^4	10^4	PBS	PBS	0.12
	10^4	10^2	PBS	PBS	0.12
	10^4	PBS	10^4	PBS	*0.03
	10^4	PBS	10^2	PBS	*0.03
	10^4	10^4	10^4	PBS	0.18
	10^4	10^2	10^2	PBS	0.18
	10^4	10^4	10^4	PBS	0.39
	10^4	10^2	10^4	PBS	0.39
SA1 10^3	10^3	PBS	PBS	PBS	*0.02
	10^3	10^4	PBS	PBS	0.07
	10^3	10^2	PBS	PBS	0.18
	10^3	PBS	10^4	PBS	*0.04
	10^3	PBS	10^2	PBS	*0.04
	10^3	10^4	10^4	PBS	0.11
	10^3	10^2	10^2	PBS	0.26
	10^3	10^4	10^3	PBS	0.62
	10^3	10^2	10^3	PBS	0.33
SA1 10^2	10^2	PBS	PBS	PBS	0.06
	10^2	10^4	PBS	PBS	0.34
	10^2	10^2	PBS	PBS	0.61
	10^2	PBS	10^4	PBS	0.16
	10^2	PBS	10^2	PBS	0.16
	10^2	10^4	10^4	PBS	0.66
	10^2	10^2	10^2	PBS	1.00
	10^2	10^4	10^2	PBS	0.34
	10^2	10^2	10^2	PBS	0.16
SA1 10^1	10^1	PBS	PBS	PBS	0.51
	10^1	10^4	PBS	PBS	0.19
	10^1	10^2	PBS	PBS	0.72
	10^1	PBS	10^4	PBS	0.77
	10^1	PBS	10^2	PBS	0.77
	10^1	10^4	10^4	PBS	0.35
	10^1	10^2	10^2	PBS	1.00
	10^1	10^4	10^1	PBS	0.51

	10^1	10^2	10^1	PBS	0.77

Yellow highlighted cells are control groups. Green highlighted cells are comparisons that are significantly different with *p*-values ≤ 0.05. Blue highlighted cells are comparisons that are trending to significance with *p*-values ranging from 0.06-0.09.

A

B

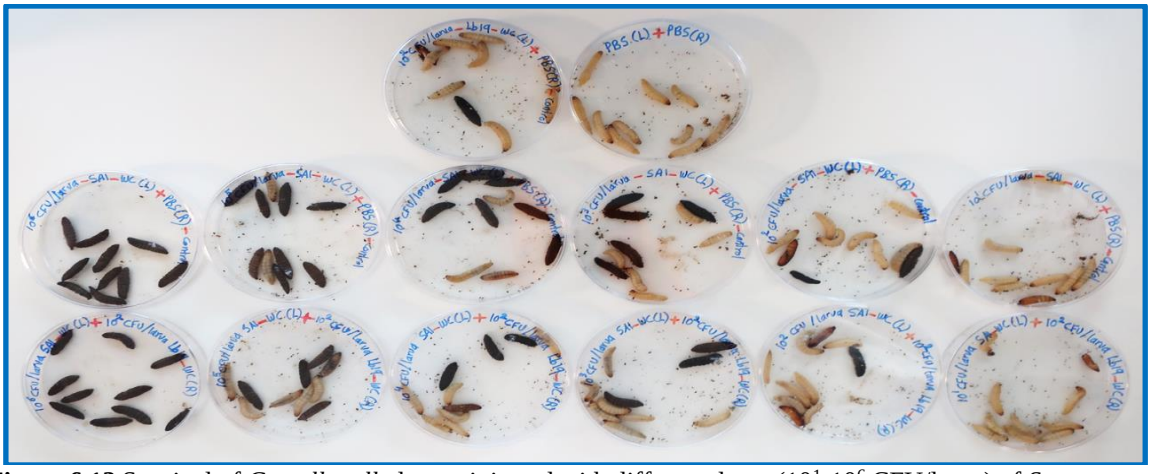

Figure 6.13 Survival of *G. mellonella* larvae injected with different doses (10^1-10^6 CFU/larva) of *S. aureus* (SA1) bacterial washed cells and treated with two doses of *Lb. delbreuckii* (Lb19) washed cells (10^4 and 10^2 CFU/larva) after six days of incubation. **A:** Groups of larvae treated with Lb19 10^4 CFU/larva. **B:** Groups of larvae treated with Lb19 10^2 CFU/larva. Within each picture: the upper left plate is the control group receiving 10 µl of either 10^4 CFU/larva (A) or 10^2 CFU/larva (B) via the left proleg and 10 µl of PBS via the right proleg, while the upper right plate is the control group receiving 10 µl of PBS via each of the prolegs. The second row of plates are control groups receiving 10 µl of each pathogenic dose via the left proleg and 10 µl of PBS via the right proleg. The third row of plates are the groups challenged with pathogenic doses and treated with Lb19 dose associated with that experiment. Each experiment A or B was conducted independently.

Table 6.3 Difference in survival percentages between two groups of larvae showing the increase and decrease in the survival. The groups are: G1, larvae co-injected with *S. aureus* (SA1) (10^1-10^6 CFU/larva) and *Lb. delbreuckii* (Lb19) (10^4 and 10^2 CFU/larva) and G2, larvae injected with the pathogenic dose alone.

SA1 dose (CFU/larva)	Survival Percentages (%)					
	Lb19 10^4 CFU/larva			Lb19 10^2 CFU/larva		
	G1: Co-injection	G2: Pathogenic dose alone	Difference in Average Survival Percentages	G1: Co-injection	G2: Pathogenic dose alone	Difference in Survival Percentages
10^6	0	0	0	0	0	0
10^5	10 (0-20)	20 (20-20)	10	35 (30-40)	20 (20-20)	15
10^4	45 (30-60)	20 (20-20)	25	45 (30-60)	20 (20-20)	25
10^3	55 (50-60)	40 (30-50)	15	65 (60-70)	40 (30-50)	25
10^2	85 (80-90)	65 (50-80)	20	90 (80-100)	65 (50-80)	25
10^1	70 (50-90)	90 (80-100)	20	90 (80-100)	90 (80-100)	0

Green highlighted cells represent the increase in survival percentages. While, blue highlighted cells represent the decrease in survival percentages. Non-highlighted cells represent no survival differences. Despite the increase in survival percentages, no significant differences were detected between the compared groups

6.4.3.2 Co-injection of *S. pyogenes* (SPA1) and *Lb. delbreuckii* (Lb19)

Evaluation of the therapeutic activity of *Lb. delbreuckii* (Lb19) against the infection of *S. pyogenes* (SPA1) was also carried out. Larvae infected with 10^1 - 10^6 CFU/larva of SPA1 were subsequently injected with the selected two doses of Lb19. Survival percentages were recorded and plotted for six days following the infection (Figure 6.14). Statistical analysis was performed to compare between survival percentages of larvae at the sixth day of infection (Table 6.4). Injection of a higher number of pathogenic cells 10^6 CFU/larva totally killed the larvae (0%). The comparison of this dose with PBS group was expected to be significant, however, it was trending towards significance with a *p*-value of 0.06. Survival of larvae infected with 10^5 CFU/larva (45%) and 10^4 CFU/larva (60%) of the SPA1 were both compared to the PBS control group (90%) and revealed significant differences with *p*-values of 0.02 and 0.05, respectively. As injected doses of the pathogen decreased (10^1 - 10^3 CFU/larva), no significant difference was obtained once compared to PBS injected larvae. The most lethal dose range of SPA1 was 10^5 - 10^6 CFU/larva because it showed only 0% - 45% of larvae survived. Lower injected doses of this pathogen had a minor lethal activity on the larvae as they revealed survival percentages more than 50%.

To assess the virulence of SPA1 and Lb19, a comparison was carried out between each dose of the pathogen and the two Lb19 doses. Although the difference between survival percentages of SPA1 10^6 CFU/larva (0%) and Lb19 10^4 CFU/larva (85%) was large, no significant difference was detected. While a difference trending to significance was found between the same pathogenic dose and Lb19 10^2 CFU/larva (90%) with a *p*-value of 0.06. Comparison of SPA1 10^5 CFU/larva (45%) with Lb19 10^4 CFU/larva (85%) and 10^2 CFU/larva (90%) revealed significant differences with *p*-values of 0.05 and 0.02, respectively. When the comparison was carried out between each of Lb19 two doses and SPA1 10^4 CFU/larva (60%), no significant difference was found for the comparison with Lb19 10^4 CFU/larva. Whereas, 10^2 CFU/larva of Lb19 showed a significant difference with a *p*-value of 0.05. Other pathogenic doses (10^1 - 10^3 CFU/larva) presented no significant differences once compared to each of the two Lb19 doses. All pathogenic doses, 10^1 - 10^6 CFU/larva revealed 0% - 85% of larvae survived which were lower than the survival percentages obtained from the two injected Lb19 doses (85% and 90%). Thus, SPA1 was more lethal than Lb19. However, 10^1 CFU/larva of SPA1 and 10^4 CFU/larva of Lb19 both revealed similar lethal effect and similar survival percentage (85%).

Co-injection of the pathogen with each Lb19 dose was performed. Groups injected with 10^6 CFU/larva of SPA1 and either 10^4 CFU/larva or 10^2 CFU/larva of Lb19 showed no survival (0%), these groups were compared to PBS injected larvae (90%). The comparison was expected to be significantly different, nevertheless, it was trending towards significance with a *p*-value of 0.06. Survival percentage of larvae co-injected with SPA1 10^5 CFU/larva and either of the two Lb19 doses was 60%. Therefore, the comparison of this percentage with PBS showed no significant difference. All other pathogenic doses (10^1 - 10^4 CFU/larva) co-injected with each of Lb19 two doses, exhibited high survival percentages ranging from 80% - 95% which were close to the survival of PBS group. For this reason, no significant differences were obtained, and all *p*-values were more than 0.05. Larvae co-injected with 10^6 CFU/larva of SPA1 and either of Lb19 doses, was the only group among all co-injected groups that showed less than 50% of larvae survived and was highly different to PBS group. Hence, this pathogenic dose was distinguished as the highest lethal dose affecting the survival of larvae.

Groups of larvae co-injected with pathogenic doses and each of Lb19 two doses were compared to the corresponding Lb19 dose injected alone. For example, SPA1 10^6 CFU/larva + Lb19 10^4 CFU/larva was compared to Lb19 10^4 CFU/larva. Although con-injection of SPA1 0^6 CFU/larva and either of Lb19 doses revealed no larvae survived (0%), when it was compared to the larvae injected with Lb19 doses alone, no significance was obtained and *p*-values were 0.10

for Lb19 10^4 CFU/larva (85%) and 0.06 for Lb19 10^2 CFU/larva (90%). As mentioned above, all survival percentages of co-injected groups contained 10^1-10^5 CFU/larva of SPA1 and either of two Lb19 doses were more than 50% (ranging from 60% - 95%). Therefore, no significant differences were found when these groups were compared to either of Lb19 two doses. Furthermore, there was slight differences (0% - 30%) between survival percentages of these co-injected groups and those obtained from the injection of Lb19 doses alone. As a result, SPA1 co-injected in any dose lower than 10^6 CFU/larva was considered a less lethal dose.

To evaluate the efficiency of Lb19 washed cells in reducing the lethal activity of SPA1 infection, group of larvae receiving a specific pathogenic dose and either of the Lb19 doses, was compared to the larvae injected with the same dose of pathogen. No larvae survived (0%) when 10^6 CFU/larva of SPA1 was injected alone or with any of Lb19 doses, thus no significant differences were obtained from the comparison of these groups. Regarding the pathogenic doses (10^1 - 10^5 CFU/larva), co-injection of these doses with each dose of Lb19 revealed higher survival percentages than those resulted from the injection of pathogen alone. The increase in survival percentages is shown in Table 6.5. It was observed that both of the Lb19 doses have a significant therapeutic effect against SPA1 infection. Larvae injected with the pathogenic doses and 10^4 CFU/larva of Lb19 showed an increase in the survival of larvae ranging from 10% - 25%. While the increase range was 10% - 20% when the pathogenic doses were co-injected with 10^2 CFU/larva of Lb19. Despite the increased survival percentages, no significant differences were detected from the statistical analysis conducted for compared groups.

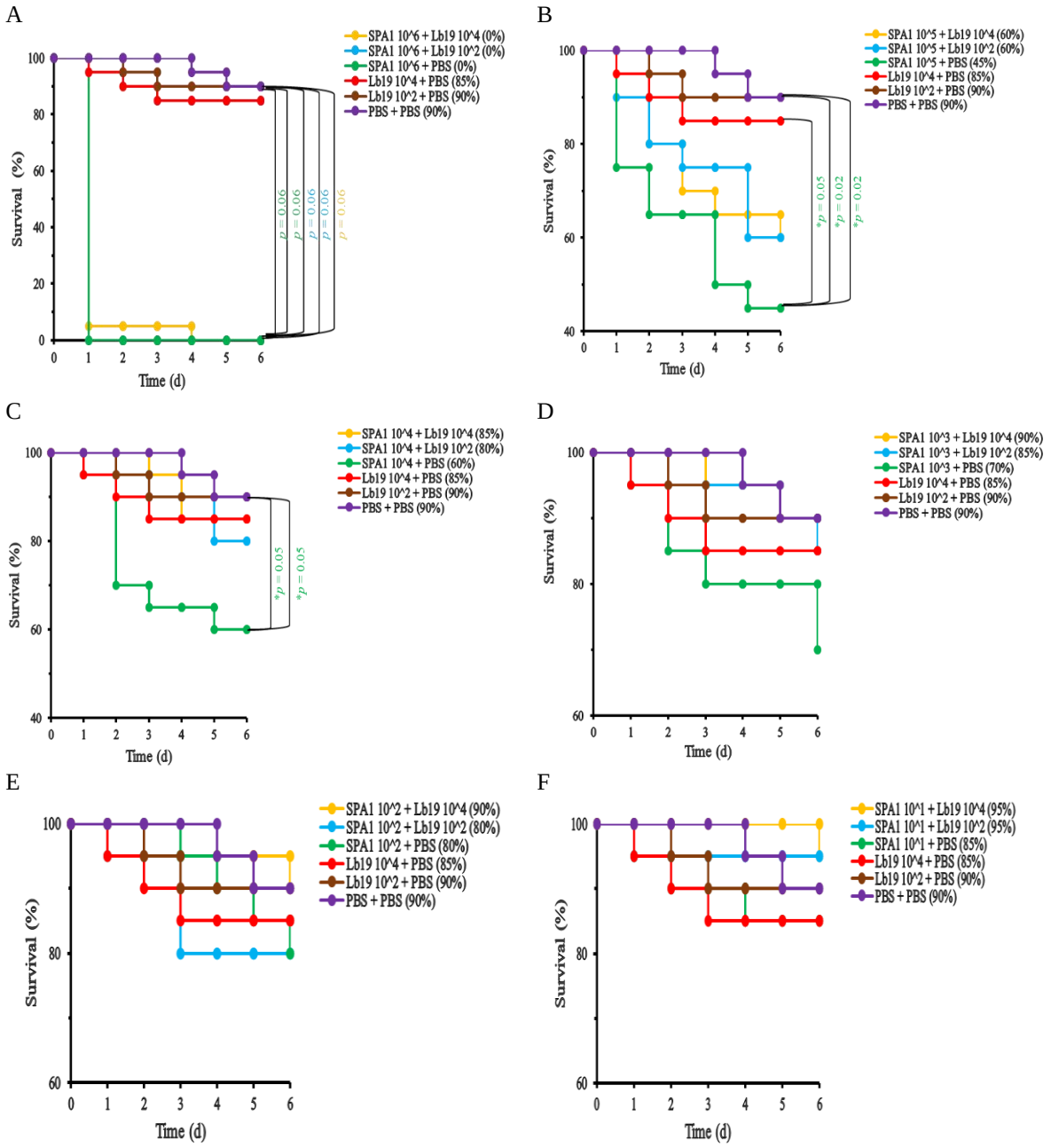

Figure 6.14 Survival plots of *G. mellonella* larvae injected with different doses (10^1-10^6 CFU/larva) of *S. pyogenes* (SPA1) bacterial washed cells and treated with two doses of *Lb. delbreuckii* (Lb19) washed cells (10^4 and 10^2 CFU/larva) through six-day incubation. Larval survival percentages at the end of experiments are given between brackets in the legends. Experiments were conducted in duplicate; and comparisons among groups were performed using non- parametric test Dunn Kruskal-Wallis test for multiple comparisons in R-Studio software. Black lines show significant differences with a p-value ≤ 0.05. **A-F:** SA1 washed cells doses. **A:** 10^6, **B:** 10^5, **C:** 10^4, **D:** 10^3, **E:** 10^2 and **F:** 10^1 CFU/larva.

Table 6.4 Significant differences as determined by non- parametric test Dunn Kruskal-Wallis test for multiple comparisons for the co-injection of *S. pyogenes* (SPA1) and *Lb. delbreuckii* (Lb19) at the sixth day post-infection.

Pathogen's dose	**Levels of compared groups SPA1 X Lb19 (10^4 and 10^2)**				***p* value**
	Left Proleg	**Right Proleg**	**Left Proleg**	**Right Proleg**	
SPA1 10^6	10^6	PBS	PBS	PBS	0.06
	10^6	10^4	PBS	PBS	0.06
	10^6	10^2	PBS	PBS	0.06
	10^6	PBS	10^4	PBS	0.10
	10^6	PBS	10^2	PBS	0.06
	10^6	10^4	10^4	PBS	0.10
	10^6	10^2	10^2	PBS	0.06
	10^6	10^4	10^6	PBS	1.00
	10^6	10^2	10^6	PBS	1.00
SPA1 10^5	10^5	PBS	PBS	PBS	*0.02
	10^5	10^4	PBS	PBS	0.14
	10^5	10^2	PBS	PBS	0.14
	10^5	PBS	10^4	PBS	*0.05
	10^5	PBS	10^2	PBS	*0.02
	10^5	10^4	10^4	PBS	0.23
	10^5	10^2	10^2	PBS	0.14
	10^5	10^4	10^5	PBS	0.44
	10^5	10^2	10^5	PBS	0.44
SPA1 10^4	10^4	PBS	PBS	PBS	*0.05
	10^4	10^4	PBS	PBS	0.72
	10^4	10^2	PBS	PBS	0.72
	10^4	PBS	10^4	PBS	0.12
	10^4	PBS	10^2	PBS	*0.05
	10^4	10^4	10^4	PBS	1.00
	10^4	10^2	10^2	PBS	0.72
	10^4	10^4	10^4	PBS	0.12
	10^4	10^2	10^4	PBS	0.12
SPA1 10^3	10^3	PBS	PBS	PBS	0.15
	10^3	10^4	PBS	PBS	0.94
	10^3	10^2	PBS	PBS	0.67
	10^3	PBS	10^4	PBS	0.28
	10^3	PBS	10^2	PBS	0.15
	10^3	10^4	10^4	PBS	0.67
	10^3	10^2	10^2	PBS	0.67
	10^3	10^4	10^3	PBS	0.13
	10^3	10^2	10^3	PBS	0.32
SPA1 10^2	10^2	PBS	PBS	PBS	0.43
	10^2	10^4	PBS	PBS	0.89
	10^2	10^2	PBS	PBS	0.62
	10^2	PBS	10^4	PBS	0.62
	10^2	PBS	10^2	PBS	0.43
	10^2	10^4	10^4	PBS	0.89
	10^2	10^2	10^2	PBS	0.62
	10^2	10^4	10^2	PBS	0.52
	10^2	10^2	10^2	PBS	0.78
SPA1 10^1	10^1	PBS	PBS	PBS	0.77
	10^1	10^4	PBS	PBS	0.77
	10^1	10^2	PBS	PBS	0.77
	10^1	PBS	10^4	PBS	1.00
	10^1	PBS	10^2	PBS	0.77
	10^1	10^4	10^4	PBS	0.55
	10^1	10^2	10^2	PBS	0.77
	10^1	10^4	10^1	PBS	0.55

	10^1	10^2	10^1	PBS	0.55

Yellow highlighted cells are control groups. Green highlighted cells are comparisons that are significantly different with *p*-values ≤ 0.05. Blue highlighted cells are comparisons that are trending to significance with *p*-values ranging from 0.06-0.09.

Table 6.5 Difference in survival percentages between two groups of larvae showing the increase in the survival. The groups are: G1, larvae co-injected with *S. pyogenes* (SPA1) (10^1-10^6 CFU/larva) and *Lb. delbreuckii* (Lb19) (10^4 and 10^2 CFU/larva) and G2, larvae injected with the pathogenic dose alone.

SPA1 dose (CFU/larva)	Survival Percentages (%)					
	Lb19 10^4 CFU/larva			Lb19 10^2 CFU/larva		
	G1: Co-injection	G2: Pathogenic dose alone	Difference in Average Survival Percentages	G1: Co-injection	G2: Pathogenic dose alone	Difference in Survival Percentages
10^6	0	0	0	0	0	0
10^5	60 (50-70)	45 (40-50)	15	60 (60-60)	45 (40-50)	15
10^4	85 (80-90)	60 (60-60)	25	80 (70-90)	60 (60-60)	20
10^3	90 (90-90)	70 (60-80)	20	85 (80-90)	70 (60-80)	15
10^2	90 (90-90)	80 (70-90)	10	80 (60-100)	80 (70-90)	0
10^1	95 (90-100)	85 (70-100)	10	95 (90-100)	85 (70-100)	10

Green highlighted cells represent the increase in survival percentages. Non-highlighted cells represent no survival differences. Despite the increase in survival percentages, no significant differences were detected between the compared groups

6.4.3.3 Co-injection of *S. dysgalactiae* subsp *equisimilis* (SDG4) and *Lb. delbreuckii* (Lb19)

The therapeutic activity of Lb19 was also tested against the infection of *S. dysgalactiae* subsp *equisimilis* (SDG4). Figure 6.15 describes the survival plots of larvae infected with four doses ranging from 10^1 - 10^4 CFU/larva of pathogenic washed cells and injected with Lb19 bacterial cells. Table 6.6 outlines the comparisons performed for groups of larvae challenged with SDG4 and Lb19 washed cells with their *p*-values.

The comparison between survival percentages of PBS injected larvae and those injected with pathogenic doses, showed that the highest injected number of pathogenic cells 10^4 CFU/larva revealed a survival percentage of 30%. Even though the difference in survival percentages between this group and PBS control group (100%) was large, the statistical difference was trending towards significance with a *p*-value of 0.07. Injection of 10^3 CFU/larva of SDG4 showed 40% of larvae survived and was significantly different to the PBS control group with a *p*-value of 0.04. Low pathogenic doses 10^2 CFU/larva and 10^1 CFU/larva presented survival percentages of 65% and 95%, respectively. These two doses were not significant when compared to PBS group, as survival percentages of the groups were close to each other. As a result, 10^3 - 10^4 CFU/larva was the dose range which showed highest levels of virulence affecting the survival of the larvae.

A comparison was also conducted between the injected doses of SDG4 and those of Lb19 to examine their lethal activity on the larvae. Although low survival percentages were obtained from the injection of the first two pathogenic doses 10^4 CFU/larva (30%) and 10^3 CFU/larva (40%), the statistical analysis revealed no significant differences between any of the four pathogenic doses and the two doses of Lb19. Regardless the absence of significance between the groups of larvae injected with SDG4 and Lb19, it was noticeable that the pathogenic doses range of 10^2 - 10^4 CFU/larva showed survival percentages (30% - 65%) lower than that of Lb19 10^2 CFU/larva (80%), hence, SDG4 injected in this dose range was more lethal. But 10^3 - 10^4 CFU/larva of SDG4 (30% - 40%) had more lethal action than Lb19 10^4 CFU/larva (65%). Furthermore, both Lb19 doses were more lethal than the lowest dose of the pathogen at 10^1 CFU/larva (95%).

Survival percentages of co-injected groups of larvae which received both SDG4 and Lb19 were also compared to the PBS group. Larvae injected with the highest number of pathogenic cells 10^4 CFU/larva followed by the injection of either 10^4 CFU/larva or 10^2 CFU/larva of Lb19, showed high death rates. Survival percentages of these groups were 5% for the first Lb19 dose and 15% for the second one. Thus, both doses were significantly different to the PBS control group with *p*-values of 0.01 and 0.05, respectively. Injection of pathogenic doses including: 10^3 CFU/larva, 10^2 CFU/larva and 10^1 CFU/larva with Lb19 10^4 CFU/larva revealed 35% of larvae survived for all doses. This survival percentage was significantly different compared to PBS with *p*-values ≤ 0.05. Whereas the same three pathogenic doses injected with Lb19 10^2 CFU/larva presented higher survival percentages which were 60%, 90% and 95%, respectively. These percentages had no significant difference when compared to PBS and the *p*-values were > 0.05. Pathogen doses (10^1 - 10^4 CFU/larva) co-injected with 10^4 CFU/larva of Lb19, in addition to the co-injected dose that contained 10^4 CFU/larva of SDG4 and 10^2 CFU/larva of Lb19 affected the survival of larvae and revealed less than 50% of larvae survived (5% - 35%). Furthermore, these survival percentages were highly different to the survival percentage of PBS group and the difference range was 65% - 95%. Therefore, these co-injected doses were all considered as the doses with the maximum virulence on the survival of larvae.

Co-injected groups with pathogenic doses and either of two Lb19 doses were compared to the corresponding dose of Lb19. For instance, SDG4 10^4 CFU/larva + Lb19 10^4 CFU/larva with Lb19 10^4 CFU/larva or SDG4 10^4 CFU/larva + Lb19 10^2 CFU/larva with Lb19 10^2 CFU/larva. All comparisons were not significantly different, except the comparison of the co-injected dose contained 10^4 CFU/larva of both the pathogen and Lb19 which revealed a low

survival percentage (5%). This co-injected dose was compared to 10^4 CFU/larva of Lb19 (65%), and the statistical difference was trending to significance with a *p*-value of 0.08. Survival percentages of larvae co-injected with 10^1 - 10^3 CFU/larva of SDG4 and 10^2 CFU/larva of Lb19 were high (60% - 95%). When these percentages were compared to the percentage resulted from the injection of Lb1910^2 CFU/larva itself (80%), the difference between percentages was slight (10% - 20%). Hence, these doses were thought to be less lethal among all co-injected doses.

The final statistical comparison was performed between co-injected groups and each pathogenic dose to investigate the therapeutic effect of Lb19 against SDG4 infection. Co-injected groups receiving any of the four pathogenic doses and 10^4 CFU/larva of Lb19 were compared to the larvae injected with that bacterial dose of pathogen. It was found that all survival percentages of the co-injected groups were lower than those of the compared pathogenic dose which means that co-injection of Lb19 at the dose of 10^4 CFU/larva affected the survival of larvae and was not effective against SDG4 infection. In terms of the second dose of Lb19, groups previously infected with either 10^3 CFU/larva and 10^2 CFU/larva and followed by the injection of 10^2 CFU/larva of Lb19, revealed higher survival percentages than those obtained from the injection of these pathogenic doses alone. The increase range in survival percentages between the compared groups was 20%-25%. Co-injection of the lowest pathogenic dose 10^1 CFU/larva, with 10^2 CFU/larva of Lb19, showed a similar survival percentage (95%) when it was compared to the pathogenic dose injected alone. As a result, it was detected that 10^2 CFU/larva of Lb19 had a protective activity against SDG4 infection, especially when the injected pathogenic doses were lower than 10^4 CFU/larva. Table 6.7 illustrates the differences in survival percentages between the co-injected groups and the group injected with the pathogen alone.

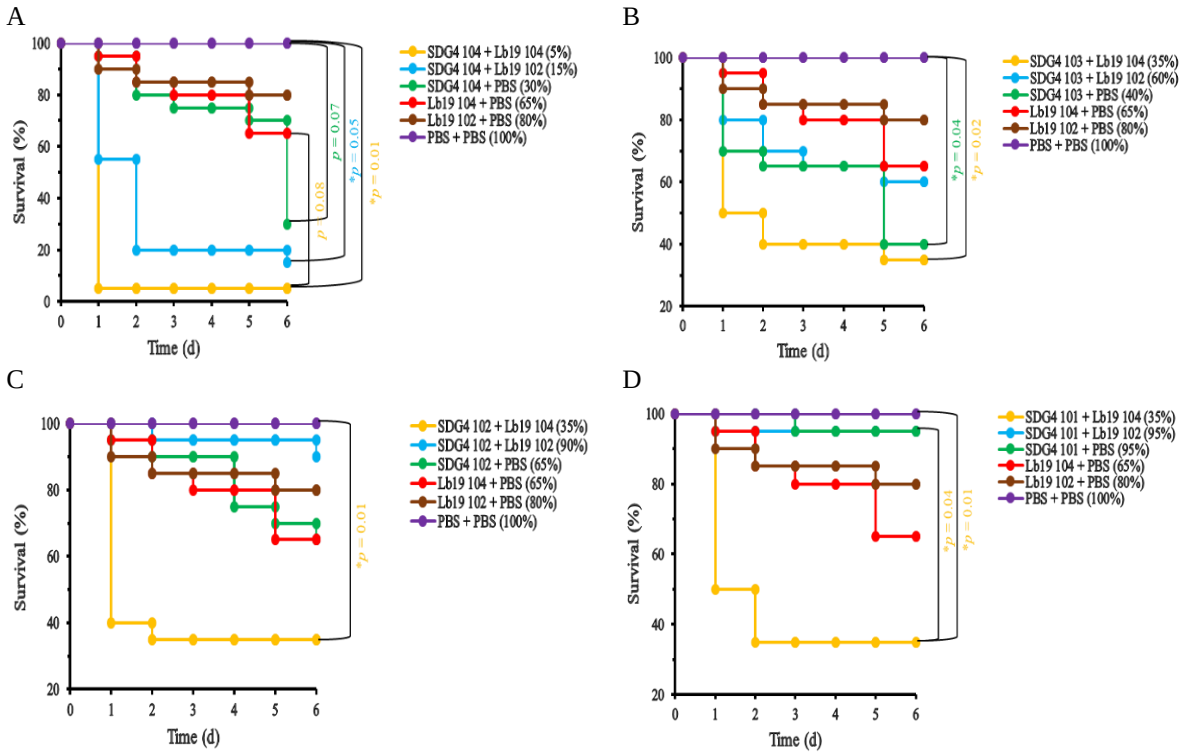

Figure 6.15 Survival plots of *G. mellonella* larvae injected with different doses (10^1-10^4 CFU/larva) of *S. dysgalactiae* subsp *equisimilis* (SDG4) bacterial washed cells and treated with two doses of *Lb. delbreuckii* (Lb19) washed cells (10^4 and 10^2 CFU/larva) through six-day incubation. Larval survival percentages at the end of experiments are given between brackets in the legends. Experiments were conducted in duplicate; and comparisons among groups were performed using non- parametric test Dunn Kruskal-Wallis test for multiple comparisons in R-Studio software. Black lines show significant differences with a p-value ≤ 0.05. **A-D:** SA1 washed cells doses. **A:** 10^4, **B:** 10^3, **C:** 10^2, **D:** 10^1 CFU/larva.

Table 6.6 Significant differences as determined by non- parametric test Dunn Kruskal-Wallis test for multiple comparisons for the co-injection of *S. dysgalactiae* subsp *equisimilis* (SDG4) and *Lb. delbreuckii* (Lb19) at the sixth day post-infection.

Pathogen's dose	**Levels of compared groups SDG4 X Lb19 (10^4 and 10^2)**				***p* value**
	Left Proleg	Right Proleg	Left Proleg	Right Proleg	
SDG4 10^4	10^4	PBS	PBS	PBS	0.07
	10^4	10^4	PBS	PBS	*0.01
	10^4	10^2	PBS	PBS	*0.05
	10^4	PBS	10^4	PBS	0.29
	10^4	PBS	10^2	PBS	0.18
	10^4	10^4	10^4	PBS	0.08
	10^4	10^2	10^2	PBS	0.14
	10^4	10^4	10^4	PBS	0.48
	10^4	10^2	10^4	PBS	0.89
SDG4 10^3	10^3	PBS	PBS	PBS	*0.04
	10^3	10^4	PBS	PBS	*0.02
	10^3	10^2	PBS	PBS	0.18
	10^3	PBS	10^4	PBS	0.29
	10^3	PBS	10^2	PBS	0.16
	10^3	10^4	10^4	PBS	0.18
	10^3	10^2	10^2	PBS	0.53
	10^3	10^4	10^3	PBS	0.78
	10^3	10^2	10^3	PBS	0.44
SDG4 10^2	10^2	PBS	PBS	PBS	0.14
	10^2	10^4	PBS	PBS	*0.01
	10^2	10^2	PBS	PBS	0.72
	10^2	PBS	10^4	PBS	1.00
	10^2	PBS	10^2	PBS	0.57
	10^2	10^4	10^4	PBS	0.29
	10^2	10^2	10^2	PBS	0.57
	10^2	10^4	10^2	PBS	0.29
	10^2	10^2	10^2	PBS	0.25
SDG4 10^1	10^1	PBS	PBS	PBS	0.61
	10^1	10^4	PBS	PBS	*0.01
	10^1	10^2	PBS	PBS	0.61
	10^1	PBS	10^4	PBS	0.25
	10^1	PBS	10^2	PBS	0.67
	10^1	10^4	10^4	PBS	0.43
	10^1	10^2	10^2	PBS	0.67
	10^1	10^4	10^1	PBS	*0.05
	10^1	10^2	10^1	PBS	1.00

Yellow highlighted cells are control groups. Green highlighted cells are comparisons that are significantly different with *p*-values ≤ 0.05. Blue highlighted cells are comparisons that are trending to significance with *p*-values ranging from 0.06-0.09.

Table 6.7 Difference in survival percentages between two groups of larvae showing the increase and decrease in the survival. The groups are: G1, larvae co-injected with *S. dysgalactiae* subsp *equisimilis* (SDG4) (10^1-10^4 CFU/larva) and *Lb. delbreuckii* (Lb19) (10^4 and 10^2 CFU/larva) and G2, larvae injected with the pathogenic dose alone.

SDG4 dose (CFU/larva)	Survival Percentages (%)					
	Lb19 10^4 CFU/larva			Lb19 10^2 CFU/larva		
	G1: Co-injection	G2: Pathogenic dose alone	Difference in Average Survival Percentages	G1: Co-injection	G2: Pathogenic dose alone	Difference in Survival Percentages
10^4	5 (0-10)	30 (10-50)	25	15 (10-20)	30 (10-50)	15
10^3	35 (30-40)	40 (20-60)	5	60 (50-70)	40 (20-60)	20
10^2	35 (20-50)	65 (60-70)	30	90 (80-100)	65 (60-70)	25
10^1	35 (20-50)	95 (90-100)	60	95 (90-100)	95 (90-100)	0

Green highlighted cells represent the increase in survival percentages. While, blue highlighted cells represent the decrease in survival percentages. Non-highlighted cells represent no survival differences. Despite the increase in survival percentages, no significant differences were detected between the compared groups

6.4.3.4 Co-injection of *S. aureus* (SA1) and *Lb. plantarum* (Lb21)

Lb. plantarum (Lb21), the second selected *Lactobacillus* species, was co-injected with both *S. aureus* (SA1) and *S. pyogenes* (SPA1). Two doses of Lb21 washed cells were chosen to be injected with the pathogens, these were 10^4 CFU/larva and 10^3 CFU/larva. Similar comparisons between injected groups were performed, as previously done for *Lb. delbreuckii* (Lb19). In terms of larvae challenged with SA1 and Lb21 (Figure 6.16 and Table 6.8), groups injected with pathogenic doses were compared to the PBS injected group. The comparison showed a large difference between survival percentages of the PBS group (80%) and each of pathogenic doses 10^6 CFU/larva (0%) and 10^5 CFU/larva (15%). However, statistical differences were trending towards significance with a *p*-value of 0.06. Injection of pathogenic doses 10^4 CFU/larva and 10^3 CFU/larva showed survival percentages of 10% and 25%, respectively. These percentages were significantly different to the PBS group with *p*-values of 0.03 for the first dose and 0.04 for the second one. After the injection of low numbers of pathogenic cells, 10^2 CFU/larva and 10^1 CFU/larva, more than 50% of larvae survived and no significant differences were found in comparison to PBS group. As a result of this comparison, 10^3 - 10^6 CFU/larva of SDG4 was described as the dose range with a maximum lethal effect on the larvae, since it revealed low survival percentages compared to PBS group, regardless the lack of significant differences between some of the compared groups.

Comparison of the lethal effect of SA1 doses and the two Lb21 doses was investigated. Injection of the highest pathogenic dose 10^6 CFU/larva revealed no survival (0%). This was compared to survival percentages obtained from the injection of Lb21 10^4 CFU/larva (70%) and 10^3 CFU/larva (85%). The comparison with the second dose showed a significant difference with a *p*-value of 0.05. Despite of the large difference between survival percentages of the pathogen and Lb21 first dose, no significant difference was obtained. Injection of the next pathogenic dose 10^5 CFU/larva resulted in 15% of larvae survived, but no significant differences were found when this dose was compared to the groups injected with both Lb21 doses. Further injected doses of SA1 including: 10^4 CFU/larva and 10^3 CFU/larva revealed 10% and 25% of larvae survived, respectively. When the first pathogenic dose was compared to Lb21 10^4 CFU/larva (70%), the difference was trending towards significance with a *p*-value of 0.08. Whereas, comparison of the second pathogenic dose with Lb21 10^4 CFU/larva (70%) showed no significance. Once the same two doses of SDG4 (10^4 CFU/larva and 10^3 CFU/larva) were compared to Lb21 10^3 CFU/larva (85%), both comparisons revealed significant differences with *p*-values of 0.02 and 0.03, respectively. Survival percentages of the groups injected with low pathogenic doses 10^1 - 10^2 CFU/larva were more than 50%, thus they were not significantly different in comparison with those of Lb21 two doses. As all pathogenic doses (10^1 - 10^6 CFU/larva) presented lower survival percentages (0% - 80%) than those of Lb21 two doses, they were more lethal than Lb21, except the lowest pathogenic dose 10^1 CFU/larva (80%) which had slightly lower lethal activity than the high dose of Lb21 10^4 CFU/larva (70%).

Co-injected groups were also compared with the PBS injected control group. Despite the large difference in survival percentages, comparison of the co-injected group receiving 10^6 CFU/larva of SA1 and either of the two Lb21 doses (0%) with the PBS injected group (80%), was trending towards significance with a *p*-value of 0.06. Co-injection of SA1 10^5 CFU/larva and Lb21 10^4 CFU/larva showed no larvae survived (0%) and was significantly different to the PBS group with a *p*-value of 0.03. When this pathogenic dose was co-injected with Lb21 10^3 CFU/larva, 20% of larvae survived and no significant difference were found in comparison to PBS. Regarding the co-injected dose contained 10^4 CFU/larva of SA1 and 10^4 CFU/larva of Lb21, it showed 40% survival. When it was compared to the PBS control group, the difference was trending towards significance with a *p*-value of 0.09. Co-injection of this pathogenic dose (10^4 CFU/larva) with the second Lb21 dose (10^3 CFU/larva) revealed 65% of larvae survived, and no significant difference was obtained in comparison to the PBS. The other three pathogenic doses co-injected with each of the two doses of Lb21, were also not significantly different to the

PBS injected larvae, since they all showed close survival percentages ranging from 45% - 85%. Despite the absence of statistically significant differences, there is a difference in survival percentages between the compared groups. Since the injection of 10^5 - 10^6 CFU/larva of SA1 and any of the two Lb21 doses obtained survival percentages (0% - 20%) lower than 50%, and these percentages were highly different to the PBS injected group (the difference was 60% - 80%). Therefore, these co-injected doses had the maximum lethal effect on the larvae among all doses.

Comparisons were also conducted between survival percentages of the groups co-injected with pathogenic doses and any of the two doses of Lb21 which were compared to the group of the corresponding Lb21 dose injected alone. For example, SA1 10^6 CFU/larva + Lb21 10^4 CFU/larva was compared to Lb21 10^4 CFU/larva. The injection of each of Lb21 doses revealed survival percentages of 70% for 10^4 CFU/larva and 85% for 10^3 CFU/larva. Co-injection of the pathogenic dose 10^6 CFU/larva and any of Lb21 doses showed a total death of larvae (0%). However, when the co-injected group was compared to the first dose of Lb21, no significant difference was obtained. Whereas comparison of the same co-injected group with the second dose of Lb21 revealed a significant difference with a *p*-value of 0.05. When 10^5 CFU/larva of SA1 was co-injected with each of the two Lb21 doses and compared to that dose of Lb21injected alone, statistical differences were trending towards significance and the *p*-values were 0.07 for Lb21 10^4 CFU/larva and 0.08 for Lb21 10^3 CFU/larva. Both of these *p*-values were anticipated to be significant as the differences between survival percentages were large ranging between 65% - 70%. The other pathogenic doses 10^1 - 10^4 CFU/larva revealed no significant differences once they were co-injected with Lb21 doses and compared to the group injected with the corresponding Lb21 dose, because the difference in survival percentages between the compared groups was small ranging between 0% - 30%. Co-injected doses contained 10^1 - 10^4 CFU/larva of SA1 and 10^3 CFU/larva of Lb21, in addition to those contained 10^1 - 10^2 CFU/larva of SA1 and 10^4 CFU/larva of Lb21 were all slightly affected the survival of larvae resulting in more than 50% survival percentages. Furthermore, a small difference (0% - 20%) was found when their survival percentages were compared to that of the larvae receiving Lb21 dose alone. Thus, these co-injected doses were recognised as the less lethal doses.

To examine the therapeutic effect of Lb21 against SA1 infection, survival percentages of the co-injected groups were compared to those of pathogenic doses. Groups of larvae which received 10^6 CFU/larva of SA1 alone or co-injected with any of Lb21 doses, all died (0%) and no significant differences were obtained from the comparison of these groups. Larvae co-

injected with 10^1 - 10^5 CFU/larva of SA1 and 10^3 CFU/larva of Lb21 showed survival percentages ranging between 20% - 85%. All these percentages were higher than those obtained from the injection of pathogenic doses alone, and the increased range was 5% - 55%. In terms of the second dose 10^4 CFU/larva of Lb21, co-injection of this dose with 10^4 CFU/larva and 10^3 CFU/larva of SA1 revealed 40% and 45% of larvae survived, respectively. Lower survival percentages resulted from the injection of these pathogenic doses which were 10% for the first pathogenic dose and 25% for the second one. Accordingly, survival percentages of the co-injected groups increased in a range of 20% - 30%. Despite this increase in survival percentages, statistical differences between the compared groups were either trending towards significance or not significant. Injection of both Lb21 doses with SA1 showed a decline in the pathogenic infection, however, 10^3 CFU/larva was more efficient than 10^4 CFU/larva. The first dose of Lb21 was able to reduce the lethal effect of the pathogen injected at the doses of 10^1 - 10^5 CFU/larva with an increase range in survival percentages was 5% - 55%. Whereas the second dose of Lb21 could reduce the infection only when the injected range of pathogenic doses was 10^3 - 10^4 CFU/larva and the increased range in survival percentages was lower ranging from 20% - 30%. Table 6.9 summarises the differences in survival percentages between co-injected groups and the groups injected with pathogenic doses alone. Figure 6.17 shows the therapeutic effect of Lb21 injected at the dose of 10^4 CFU/larva against *S. aureus* infection.

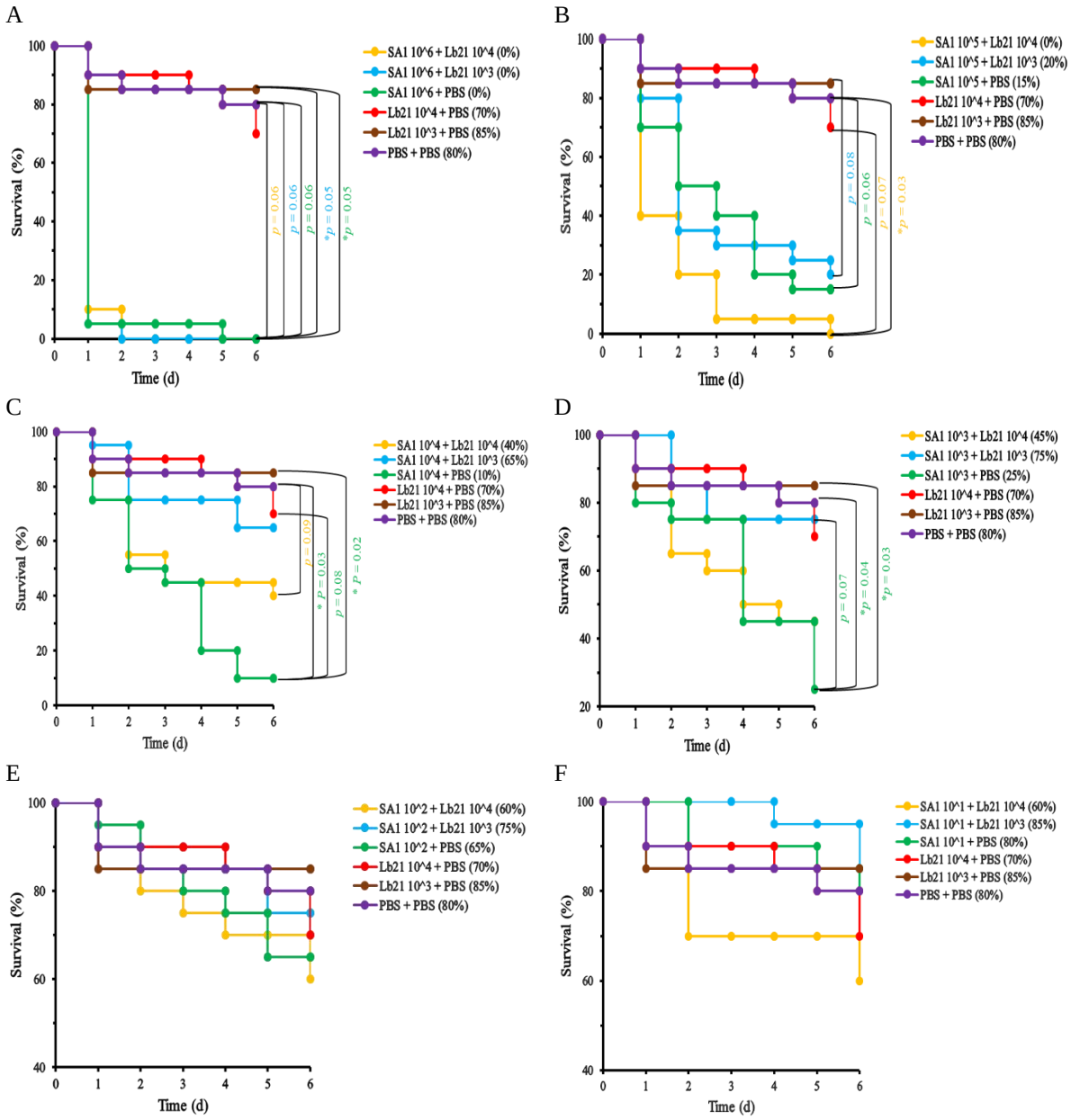

Figure 6.16 Survival plots of *G. mellonella* larvae injected with different doses (10^1-10^6 CFU/larva) of S. aureus (SA1) bacterial washed cells and treated with two doses of *Lb. plantarum* (Lb21) washed cells (104 and 10^3 CFU/larva) through six days of incubation. Larval survival percentages at the end of experiments are given between brackets in the legends. Experiments were conducted in duplicate; and comparisons among groups were performed using non- parametric test Dunn Kruskal-Wallis test for multiple comparisons in R-Studio software. Black lines show significant differences with a p-value ≤ 0.05. A-F: SA1 washed cells doses. A: 10^6, B: 10^5, C: 10^4, D: 10^3, E: 10^2 and F: 10^1 CFU/larva.

Table 6.8 Significant differences as determined by non- parametric test Dunn Kruskal-Wallis test for multiple comparisons for the co-injection of *S. aureus* (SA1) and *Lb. plantarum* (Lb21) at the sixth day post-infection.

Pathogen's dose	Levels of compared groups SA1 X Lb21 (10^4 and 10^3)				p value
	Left Proleg	Right Proleg	Left Proleg	Right Proleg	
SA1 106	10^6	PBS	PBS	PBS	0.06
	10^6	10^4	PBS	PBS	0.06
	10^6	10^3	PBS	PBS	0.06
	10^6	PBS	10^4	PBS	0.14
	10^6	PBS	10^3	PBS	0.05
	10^6	10^4	10^4	PBS	0.14
	10^6	10^3	10^3	PBS	0.05
	10^6	10^4	10^6	PBS	1.00
	10^6	10^3	10^6	PBS	1.00
SA1 105	10^5	PBS	PBS	PBS	0.06
	10^5	10^4	PBS	PBS	0.03
	10^5	10^3	PBS	PBS	0.11
	10^5	PBS	10^4	PBS	0.26
	10^5	PBS	10^3	PBS	0.11
	10^5	10^4	10^4	PBS	0.07
	10^5	10^3	10^3	PBS	0.08
	10^5	10^4	10^5	PBS	0.48
	10^5	10^3	10^5	PBS	0.89
SA1 104	10^4	PBS	PBS	PBS	0.03
	10^4	10^4	PBS	PBS	0.09
	10^4	10^3	PBS	PBS	0.44
	10^4	PBS	10^4	PBS	0.08
	10^4	PBS	10^3	PBS	0.02
	10^4	10^4	10^4	PBS	0.23
	10^4	10^3	10^3	PBS	0.36
	10^4	10^4	10^4	PBS	0.58
	10^4	10^3	10^4	PBS	0.14
SA1 103	10^3	PBS	PBS	PBS	0.04
	10^3	10^4	PBS	PBS	0.12
	10^3	10^3	PBS	PBS	0.83
	10^3	PBS	10^4	PBS	0.12
	10^3	PBS	10^3	PBS	0.03
	10^3	10^4	10^4	PBS	0.26
	10^3	10^3	10^3	PBS	0.73
	10^3	10^4	10^3	PBS	0.67
	10^3	10^3	10^3	PBS	0.07
SA1 102	10^2	PBS	PBS	PBS	0.22
	10^2	10^4	PBS	PBS	0.15
	10^2	10^3	PBS	PBS	0.83
	10^2	PBS	10^4	PBS	0.61
	10^2	PBS	10^3	PBS	0.17
	10^2	10^4	10^4	PBS	0.47
	10^2	10^3	10^3	PBS	0.72
	10^2	10^4	10^2	PBS	0.83
	10^2	10^3	10^2	PBS	0.31
SA1 101	10^1	PBS	PBS	PBS	1.00
	10^1	10^4	PBS	PBS	0.39
	10^1	10^3	PBS	PBS	0.61
	10^1	PBS	10^4	PBS	0.47
	10^1	PBS	10^3	PBS	0.83
	10^1	10^4	10^4	PBS	0.89
	10^1	10^3	10^3	PBS	0.77
	10^1	10^4	10^1	PBS	0.39

	10^1	10^3	10^1	PBS	0.61

Yellow highlighted cells are control groups. Green highlighted cells are comparisons that are significantly different with *p*-values ≤ 0.05. Blue highlighted cells are comparisons that are trending to significance with *p*-values ranging from 0.06-0.09.

Table 6.9 Difference in survival percentages between two groups of larvae showing the increase and decrease in the survival. The groups are: G1, larvae co-injected with *S. aureus* (SA1) (10^1-10^6 CFU/larva) and *Lb. plantarum* (Lb21) (10^4 and 10^3 CFU/larva) and G2, larvae injected with the pathogenic dose alone.

SA1 dose (CFU/larva)	Survival Percentages (%)					
	Lb21 10^4 CFU/larva			Lb21 10^3 CFU/larva		
	G1: Co-injection	G2: Pathogenic dose alone	Difference in Average Survival Percentages	G1: Co-injection	G2: Pathogenic dose alone	Difference in Survival Percentages
10^6	0	0	0	0	0	0
10^5	0	15 (10-20)	15	20 (0-40)	15 (10-20)	5
10^4	40 (30-50)	10 (0-20)	30	65 (60-70)	10 (0-20)	55
10^3	45 (40-50)	25 (10-40)	20	75 (70-80)	25 (10-40)	50
10^2	60 (50-70)	65 (60-70)	5	75 (70-80)	65 (60-70)	10
10^1	60 (40-80)	80 (80-80)	20	85 (80-90)	80 (80-80)	5

Green highlighted cells represent the increase in survival percentages. While, blue highlighted cells represent the decrease in survival percentages. Non-highlighted cells represent no survival differences. Despite the increase in survival percentages, no significant differences were detected between the compared groups

Figure 6.17 Survival of *G. mellonella* larvae injected with different doses (10^1-10^6 CFU/larva) of *S. aureus* (SA1) bacterial washed cells and treated with10^4 CFU/larva of *Lb. plantarum* (Lb21) washed cells after six days of incubation. The upper left plate is the control group receiving 10 µl of 10^4 CFU/larva via the left proleg and 10 µl of PBS via the right proleg, while the upper right plate is the control group receiving 10 µl of PBS via each of the prolegs. The second row of plates are control groups receiving 10 µl of each pathogenic dose via the left proleg and 10 µl of PBS via the right proleg. The third row of plates are the groups challenged with pathogenic doses and Lb21 dose.

6.4.3.5 Co-injection of *S. pyogenes* (SPA1) and *Lb. plantarum* (Lb21)

The six pathogenic doses of *S. pyogenes* (SPA1) were also co-injected with Lb21 two doses. Survival plots of the co-injected groups are shown in Figure 6.18, while statistical differences obtained from the compared co-injected groups are illustrated in Table 6.10. Survival percentages of larvae injected with SPA1 doses were compared to that of the PBS injected group. The highest number of pathogenic cells (10^6 CFU/larva) revealed no larvae survived (0%), therefore it was significantly different to the PBS group (95%) with a *p*-value of 0.02. The pathogenic dose 10^5 CFU/larva presented higher survival percentage (30%) than the previous dose. Although this percentage was lower than that of the PBS control group, the statistical difference was trending towards significance with a *p*-value of 0.06. Further, lower pathogenic doses 10^4 CFU/larva and 10^3 CFU/larva revealed high survival percentages which were 80% and 75%, respectively. Since these survival percentages were close to the survival of the PBS injected larvae, no significant differences were obtained. An increase in survival percentages of larvae was expected by decreasing the number of injected pathogenic cells. Nevertheless, injection of low pathogenic doses 10^2 CFU/larva and 10^1 CFU/larva presented a decline in survival percentages to 50% and 60%, respectively. Thus, comparison of these percentages with the PBS group, showed significant differences with *p*-values of 0.02 for the first percentage and 0.01 for the second one. As a result, SPA1 revealed the maximum virulent effect on the larvae when it was injected at the doses range of 10^5 - 10^6 CFU/larva, since these injected doses showed 0% - 30% of larvae survived.

The lethal effect of SPA1 doses and each of Lb21 two doses was compared. A difference in survival percentages was found between the larvae injected with 10^6 CFU/larva of SPA1 (0%) and the groups receiving either 10^4 CFU/larva (65%) or 10^3 CFU/larva (85%) of Lb21. However, no significant difference was detected for the comparison with Lb21 first dose, while the difference was trending towards significance with a *p*-value of 0.06 for the comparison with Lb21 second dose. Injection of pathogenic doses 10^5 CFU/larva, 10^4 CFU/larva and 10^3 CFU/larva revealed 30%, 80% and 75% of larvae survived, respectively. No significant differences were observed when these pathogenic doses were compared to survival percentages of each of Lb21 doses and all *p*-values obtained from the statistical analysis were > 0.05. Because of the sudden decrease in survival percentages after the injection of lower doses of SPA1 including: 10^2 CFU/larva (50%) and 10^1 CFU/larva (60%), comparison of both pathogenic doses with Lb21 10^3 CFU/larva (85%) displayed statistical differences trending to significance with a *p*-value of 0.09. Whereas, no significant differences were found for the comparison of these

pathogenic doses with Lb21 10^4 CFU/larva (65%), since all survival percentages were close to each other. SPA1 had a higher lethal activity than the dose 10^3 CFU/larva of Lb21, as all pathogenic doses (10^1 - 10^6 CFU/larva) revealed survival percentages (0% - 80%) lower than that obtained from the injection of Lb21 in this dose (85%). In terms of 10^4 CFU/larva of Lb21 that showed slightly low survival percentage (65%), only higher doses of SPA1 (10^5 - 10^6 CFU/larva) were more lethal than this dose of Lb21.

Co-injected groups of larvae were also compared to PBS. Survival percentages of larvae co-injected with the highest dose of SPA1 and each of Lb21 two doses were significantly different to PBS injected larvae, with a *p*-value of 0.02 for both doses. Co-injection of pathogenic doses 10^5 CFU/larva and 10^4 CFU/larva with Lb21 10^4 CFU/larva showed survival percentages of 30% and 65%, respectively. These percentages were trending towards significance once compared to PBS and the *p*-value was 0.06 for both pathogenic doses. The same two pathogenic doses co-injected with Lb21 10^3 CFU/larva revealed no larvae survived (0%) for the first pathogenic dose and 15% survival percentage for the second one. Therefore, they were both significantly different to the PBS injected group with a similar *p*-value of 0.01. Regarding the next two doses of SPA1 10^3 CFU/larva and 10^2 CFU/larva, co-injection of these with each of Lb21 two doses, presented survival percentages ranging between 40% - 55%. All comparisons of these percentages with the PBS group were significantly different and the *p*-values were $\leq$ 0.05. The lowest dose of SPA1 (10^1 CFU/larva) co-injected with Lb21 10^4 CFU/larva revealed 70% of larvae survived and was not significantly different in comparison to PBS. Whereas the co-injection of this pathogenic dose with Lb21 10^3 CFU/larva showed 65% of larvae survived and a significant difference with a *p*-value of 0.05 compared to PBS. Co-injected doses that showed survival percentages lower than 50% which differed to the survival percentage of PBS group in a rate more than 50%, were distinguished as the doses with the maximum virulence on the larval survival. These doses were 10^3 - 10^6 CFU/larva of SPA1 co-injected with 10^3 CFU/larva of Lb21 and 10^5 - 10^6 of the pathogen co-injected with 10^4 CFU/larva of Lb21.

The next comparison was carried out between survival percentages of co-injected groups and each of the two Lb21 doses. For instance, a comparison between SPA1 10^6 CFU/larva co-injected with Lb21 10^4 CFU/larva and Lb21 10^4 CFU/larva alone. Survival percentages of larvae co-injected with any of pathogenic doses and Lb21 10^4 CFU/larva were compared to that of the group injected with this dose of Lb21. All statistical differences resulted from this comparison were not significant. No larvae survived for the groups co-injected with Lb21 10^3 CFU/larva and either 10^6 CFU/larva or 10^5 CFU/larva of SPA1 (0%). Whereas 85% of larvae survived when

Lb21 injected in this dose alone. Although the difference between these two survival percentages was large, the statistical analysis was trending towards significance with a *p*-value of 0.06 when Lb21 injected with the first pathogenic dose, while the difference was significant with a *p*-value of 0.02 when Lb21 injected with the second pathogenic dose. Co-injection of the next lower pathogenic doses 10^4 CFU/larva and 10^3 CFU/larva with Lb21 10^3 CFU/larva presented 15% and 40% of larvae survived, respectively. Comparisons of these percentages with the survival percentage of the group injected with Lb21 dose alone, revealed significant differences with *p*-values of 0.03 for the first percentage and 0.04 for the second one. Half of the larvae (50%) survived after the co-injection of SPA1 10^2 CFU/larva and Lb21 10^3 CFU/larva, a difference trending towards significance with a *p*-value of 0.09 was obtained after the comparison of this group with the larvae injected with Lb21 dose alone. The co-injected dose contained 10^1 CFU/larva of SPA1 and the same dose of Lb21 revealed a survival of 65%, which was not significantly different to the larvae injected with Lb21 alone because there is no large difference between the percentages of survival. Depending on these results, it was found that co-injected doses, with a less lethal effect on the larvae, were 10^1 - 10^4 CFU/larva of SPA1 co-injected with 10^4 CFU/larva of Lb21, and 10^1 - 10^2 CFU/larva of SPA1 co-injected with 10^3 CFU/larva of Lb21 as well. All these co-injected doses shared two characters including their high survival percentages that were equal or more than 50%, and the slight difference between their survival percentages and that of Lb21 doses which approached only 0% - 35%.

The last comparison was performed to evaluate the therapeutic activity of Lb21 two doses against the infection of SPA1. As most of survival percentages of co-injected groups were lower than those resulting from the injection of SPA1 doses alone. Thus, no significant differences were obtained, and all *p*-values were > 0.05. However, the comparison of groups infected with low pathogenic doses including 10^2 CFU/larva and 10^1 CFU/larva with groups receiving these pathogenic doses and any of Lb21 doses, showed that survival percentages of co-injected larvae either slightly increased with a rate of 5% - 10% or remained the same (Table 6.11). Consequently, both Lb21 doses had no effect against SPA1 infection but were able to decrease the lethal activity of the pathogen injected in low doses. Figure 6.19 displays the effect of Lb21 two doses against different doses of *S. pyogenes*.

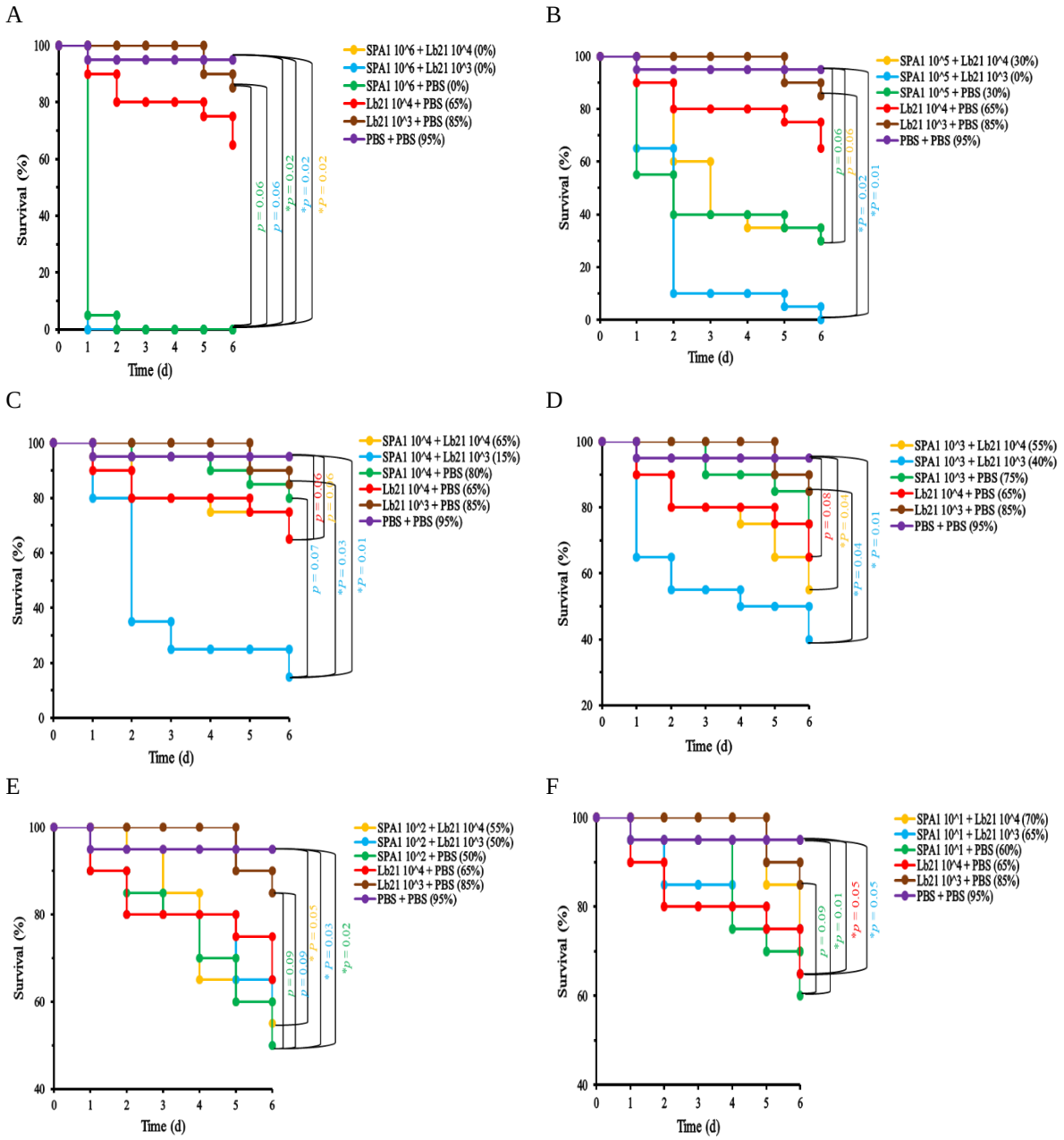

Figure 6.18 Survival plots of *G. mellonella* larvae injected with different doses (10^1-10^6 CFU/larva) of *S. pyogenes* (SPA1) bacterial washed cells and treated with two doses of *Lb. plantarum* (Lb21) washed cells (10^4 and 10^3 CFU/larva) through six days of incubation. Larval survival percentages at the end of experiments are given between brackets in the legends. Experiments were conducted in duplicate; and comparisons among groups were performed using non- parametric test Dunn Kruskal-Wallis test for multiple comparisons in R-Studio software. Black lines show significant differences with a p-value ≤ 0.05. **A-F:** SA1 washed cells doses. **A:** 10^6, **B:** 10^5, **C:** 10^4, **D:** 10^3, **E:** 10^2 and **F:** 10^1 CFU/larva.

Table 6.10 Significant differences as determined by non- parametric test Dunn Kruskal-Wallis test for multiple comparisons for the co-injection of *S. pyogenes* (SPA1) and *Lb. plantarum* (Lb21) at the sixth day post-infection.

Pathogen's doze	**Levels of compared pairs SPA1 X Lb21 (10^4 and 10^3)**				***p* value**
	Left Proleg	**Right Proleg**	**Left Proleg**	**Right Proleg**	
SPA1 10^6	10^6	PBS	PBS	PBS	0.02
	10^6	10^4	PBS	PBS	0.02
	10^6	10^3	PBS	PBS	0.02
	10^6	PBS	10^4	PBS	0.24
	10^6	PBS	10^3	PBS	0.06
	10^6	10^4	10^4	PBS	0.24
	10^6	10^3	10^3	PBS	0.06
	10^6	10^4	10^6	PBS	1.00
	10^6	10^3	10^6	PBS	1.00
SPA1 10^5	10^5	PBS	PBS	PBS	0.06
	10^5	10^4	PBS	PBS	0.06
	10^5	10^3	PBS	PBS	0.01
	10^5	PBS	10^4	PBS	0.40
	10^5	PBS	10^3	PBS	0.14
	10^5	10^4	10^4	PBS	0.40
	10^5	10^3	10^3	PBS	0.02
	10^5	10^4	10^5	PBS	1.00
	10^5	10^3	10^5	PBS	0.40
SPA1 10^4	10^4	PBS	PBS	PBS	0.36
	10^4	10^4	PBS	PBS	0.06
	10^4	10^3	PBS	PBS	0.01
	10^4	PBS	10^4	PBS	0.33
	10^4	PBS	10^3	PBS	0.73
	10^4	10^4	10^4	PBS	1.00
	10^4	10^3	10^3	PBS	0.03
	10^4	10^4	10^4	PBS	0.33
	10^4	10^3	10^4	PBS	0.07
SPA1 10^3	10^3	PBS	PBS	PBS	0.26
	10^3	10^4	PBS	PBS	0.04
	10^3	10^3	PBS	PBS	0.01
	10^3	PBS	10^4	PBS	0.53
	10^3	PBS	10^3	PBS	0.53
	10^3	10^4	10^4	PBS	0.78
	10^3	10^3	10^3	PBS	0.04
	10^3	10^4	10^3	PBS	0.36
	10^3	10^3	10^3	PBS	0.14
SPA1 10^2	10^2	PBS	PBS	PBS	0.02
	10^2	10^4	PBS	PBS	0.05
	10^2	10^3	PBS	PBS	0.03
	10^2	PBS	10^4	PBS	0.78
	10^2	PBS	10^3	PBS	0.09
	10^2	10^4	10^4	PBS	0.68
	10^2	10^3	10^3	PBS	0.09
	10^2	10^4	10^2	PBS	0.89
	10^2	10^3	10^2	PBS	0.73
SPA1 10^1	10^1	PBS	PBS	PBS	0.01
	10^1	10^4	PBS	PBS	0.17
	10^1	10^3	PBS	PBS	0.05
	10^1	PBS	10^4	PBS	0.56
	10^1	PBS	10^3	PBS	0.09

	10^1	10^4	10^4	PBS	0.56
	10^1	10^3	10^3	PBS	0.13
	10^1	10^4	10^1	PBS	0.25
	10^1	10^3	10^1	PBS	0.56

Yellow highlighted cells are control groups. Green highlighted cells are comparisons that are significantly different with *p*-values ≤ 0.05. Blue highlighted cells are comparisons that are trending to significance with *p*-values ranging from 0.06-0.09.

Table 6.11 Difference in survival percentages between two groups of larvae showing the increase and decrease in the survival. The groups are: G1, larvae co-injected with *S. pyogenes* (SPA1) (10^1-10^6 CFU/larva) and *Lb. plantarum* (Lb21) (10^4 and 10^3 CFU/larva) and G2, larvae injected with the pathogenic dose alone.

SPA1 dose (CFU/larva)	Survival Percentages (%)					
	Lb21 10^4 CFU/larva			Lb21 10^3 CFU/larva		
	G1: Co-injection	G2: Pathogenic dose alone	Difference in Average Survival Percentages	G1: Co-injection	G2: Pathogenic dose alone	Difference in Survival Percentages
10^6	0	0	0	0	0	0
10^5	30 (20-40)	30 (30-30)	0	0	30 (30-30)	30
10^4	65 (60-70)	80 (80-80)	15	15 (10-20)	80 (80-80)	65
10^3	55 (40-70)	75 (70-80)	20	40 (30-50)	75 (70-80)	35
10^2	55 (40-70)	50 (20-80)	5	50 (50-50)	50 (20-80)	0
10^1	70 (70-70)	60 (60-60)	10	65 (60-70)	60 (60-60)	5

Green highlighted cells represent the increase in the survival percentages. While, blue highlighted cells represent the decrease in the survival percentages. Non-highlighted cells represent no survival differences. Despite the increase in survival percentages, no significant differences were detected between the compared groups

A

B

Figure 6.19 Survival of *G. mellonella* larvae injected with different doses (10^1-10^6 CFU/larva) of *S. pyogenes* (SPA1) bacterial washed cells and treated with two doses of *Lb. plantarum* (Lb21) washed cells (10^4 and 10^3 CFU/larva) after six days of incubation. **A:** Groups of larvae treated with Lb21 10^4 CFU/larva. **B:** Groups of larvae treated with Lb21 10^3 CFU/larva. Within each picture: the upper left plate is the control group receiving 10 µl of either 10^4 CFU/larva (A) or 10^3 CFU/larva (B) via the left proleg and 10 µl of PBS via the right proleg, while the upper right plate is the control group receiving 10 µl of PBS via each of the prolegs. The second row of plates are control groups receiving 10 µl of each pathogenic dose via the left proleg and 10 µl of PBS via the right proleg. The third row of plates are the groups challenged with pathogenic doses and treated with Lb19 dose associated with that experiment. Each experiment A or B was conducted independently.

6.4.4 Topical Application of the Pathogen and *Lactobacillus* Species

The Infection of larvae with *S. pyogenes* (SPA1) was performed by the topical application of pathogenic washed cells to the larvae dorsum. Bacterial cells were applied at the doses ranging from 10^1 - 10^4 CFU/larva. The infected larvae were treated with the washed cells solution of *Lb. plantarum* (Lb21) that contained 10^6 CFU/larva, and with the undiluted CFS of the same species. This experiment was conducted to evaluate the effectiveness of the topical application as a technique used for *G. mellonella* infection with skin pathogens. Infection trials undertaken in the present study showed that the direct application of the pathogen to the intact cuticle of the larvae was not a successful method of infection, since the larvae remained alive with no obvious signs

of infection in the inoculated area. Whereas the application of pathogenic doses to the scratched cuticle caused an injury and stimulated the immune response and the production of melanin, forming several black separate scratches in the area for mild injured larvae (S1) or a black lesion for severely injured ones (S2). As melanization is the first indication of infection in the wax worm, it occurred due to the scratch step which enable the bacterial inoculum to enter through the damaged cuticle and caused the infection. Therefore, all groups of larvae were scratched prior to the topical application of the bacterial inoculum. Figure 6.20 shows the topical application of *S. aureus* (SA1) and *S. pyogenes* (SPA1) with and without scratching the inoculated area.

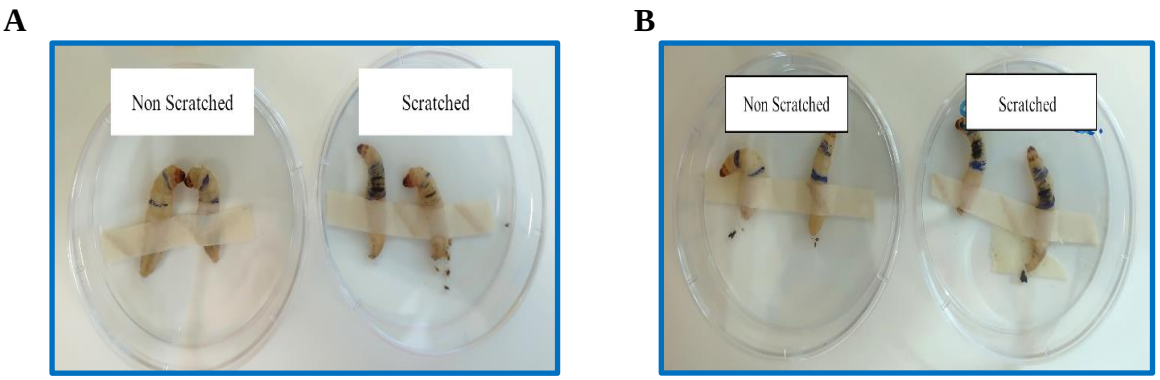

Figure 6.20 Topical Application of pathogenic cells to the larval dorsum showing the absence of infection for the non-scratched larvae (Left plate) and the presence of injury for the scratched larvae (Right plate) of each picture. A: *Staphylococcus aureus* (SA1) and B: *Streptococcus pyogenes* (SPA1)

Table 6.12 depicts the percentages of larvae after the topical infection of SPA1 and the treatment with Lb21 showing different injury scores for control and experimental groups through six days of incubation. Control group of larvae topically administered with PBS showed 10% (S3) of larvae died after one day of incubation, which increased into 20% on the third day. During the last three days of incubation, no more death was observed for the PBS group and 80% of larvae remained alive. This percentage of survived larvae was consistent included 10% of larvae with no injury (S0), 60% mildly injured (S1), 10% got severe injury (S2). Regarding the larvae topically administered with 10^6 CFU/larva of Lb21 bacterial cells, all the larvae remained alive included 80% with mild injury (S1) and 20% severely injured (S2). When the undiluted CFS of Lb21 was applied, 60% of larvae got a mild injury (S1) and 40% died (S3). With respect to pathogenic doses, application of the highest dose 10^4 CFU/larva of SPA1 resulted in all larvae died (S3 = 100%) on the fifth day of the infection. This was the maximum percentage of death

obtained among all four control groups at the end of experiment. Both applied doses of pathogenic washed cells 10^3 CFU/larva and 10^2 CFU/larva revealed a death percentage of 60% and 40% of larvae survived. It was observed that by decreasing the pathogenic dose applied to the larvae, the percentage of dead larvae decreased approaching to 30% for the lowest pathogenic dose 10^1 CFU/larva.

The death percentage of the larvae applied with each dose of the pathogen was compared to that of the larvae infected with the same pathogenic dose and treated with both Lb21 washed cells and CFS. While the larvae infected with 10^4 CFU/larva of SPA1 all died (S3 = 100%), the group which was applied with this pathogenic dose and treated with Lb21 cells showed only 20% of larvae died. Whereas the treatment with the CFS revealed a death percentage of 60% indicating that the survival of larvae increased for both treated groups compared to the untreated group. However, treatment with the CFS of Lb21 presented higher death percentage than that of the group treated with bacterial cells. In terms of the pathogenic dose 10^3 CFU/larva, death percentages of the larvae infected with this dose and the larvae treated with the CFS were similar (60%), while this percentage was 50% for the cells-treated group. Regarding the next two doses of the pathogen 10^2 CFU/larva and 10^1 CFU/larva, the death percentages of infected larvae were 60% and 30%, respectively. Both percentages decreased to 10% of larvae died after the treatment with Lb21 cells. In contrast, larvae infected with these two pathogenic doses and treated with the CFS showed an increase in the percentages of death which approached to 70% for the larvae infected with 10^2 CFU/larva of SPA1, and 90% for the larvae infected with 10^1 CFU/larva. In general, treatment with the washed cells of Lb21 was more effective than the treatment with the CFS as the death percentages of all larval groups treated with bacterial cells were lower in comparison with the untreated larvae. Whereas the treatment with the CFS of Lb21 caused more death for the groups infected with the two lower pathogenic doses showing that the application of the CFS over the infected area affected the survival of larvae. Figure 6.21 displays the topical application of different doses of *S. pyogenes* (SPA1) to the larval dorsum topically treated with *Lb. plantarum* (Lb21) bacterial cells and cell free supernatant.

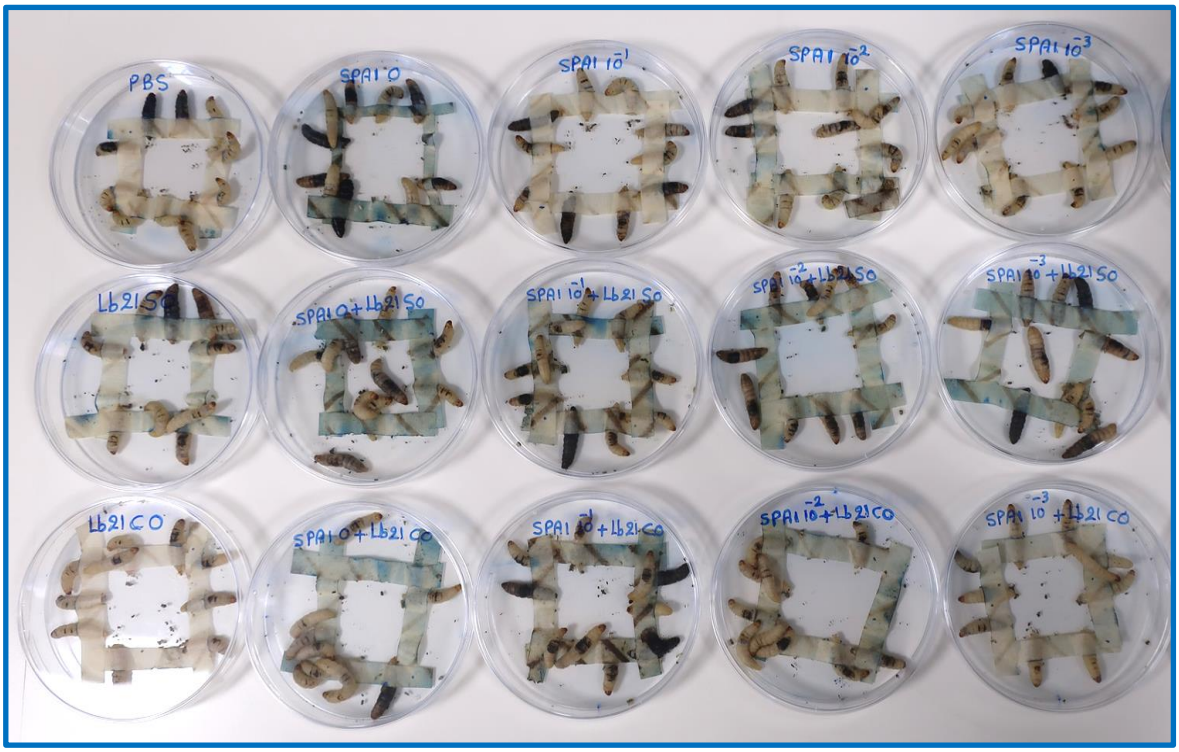

Figure 6.21 The topical application of different doses (101-104 CFU/larva) of *S. pyogenes* (SPA1) washed cells to the larval dorsum and the topical treatment with 106 CFU/larva of *Lb. plantarum* (Lb21) washed cells (C0) and undiluted cell free supernatant (S0). The first row: the left plate is the control group applied with 10 µl of PBS and the rest four plates are control groups applied with 10 µl of SPA1 doses from left to right (104, 103, 102 and 101 CFU/larva). The second row: The left plate is the control group applied with 10 µl of undiluted supernatant and the rest four plates are the larvae applied with pathogenic doses and treated with undiluted supernatant. The third row: The left plate is the control group applied with 10 µl of 106 CFU/larva of Lb. plantarum (Lb21) washed cells and the rest four plates are the larvae applied with pathogenic doses and treated with bacterial washed cells.

Table 6.12 The percentages of *G. mellonella* larvae topically infected with different doses (10^1-10^4 CFU/larva) of *S. pyogenes* (SPA1) bacterial washed cells and topically treated with *Lb. plantarum* (Lb21) washed cells (10^6 CFU/larva) and cell free supernatant (CFS), showing the injury severity scores (ISS) for experimental and control groups through six days of incubation. The symbols of S0, S1, S2 and S3 demonstrate the absence of injury, mild injury, severe injury and dead larvae, respectively.

Treatment	Percentages of Larvae																											
	Day 0				Day 1				Day 2				Day 3				Day 4				Day 5				Day 6			
	S0	S1	S2	S3	S0	S1	S2	S3	S0	S1	S2	S3	S0	S1	S2	S3	S0	S1	S2	S3	S0	S1	S2	S3	S0	S1	S2	S3
SPA1 10^4	100	0	0	0	40	10	20	30	10	30	10	50	0	40	10	50	0	40	10	50	0	0	0	100	0	0	0	100
SPA1 10^4 + Lb21 10^6	100	0	0	0	0	100	0	0	0	90	0	10	0	90	0	10	0	90	0	10	0	80	10	10	0	70	10	20
SPA1 10^4 + Lb21 CFS	100	0	0	0	0	70	20	10	0	60	20	20	0	50	20	30	0	50	20	30	0	50	10	40	0	30	10	60
SPA1 10^3	100	0	0	0	20	30	30	20	0	50	10	40	0	50	0	50	0	50	0	50	0	50	0	50	0	40	0	60
SPA1 10^3 + Lb21 10^6	100	0	0	0	0	10	70	20	0	10	60	30	0	10	60	30	0	10	60	30	0	10	60	30	0	10	40	50
SPA1 10^3 + Lb21 CFS	100	0	0	0	0	60	30	10	0	60	30	10	0	50	40	10	0	50	40	10	0	50	30	20	0	20	20	60
SPA1 10^2	100	0	0	0	0	30	60	10	0	40	0	60	0	40	0	60	0	40	0	60	0	40	0	60	0	30	10	60
SPA1 10^2 + Lb21 10^6	100	0	0	0	0	80	20	0	0	80	20	0	0	80	20	0	0	80	20	0	0	70	20	10	0	70	20	10
SPA1 10^2 + Lb21 CFS	100	0	0	0	0	40	20	40	0	40	20	40	0	40	10	50	0	40	10	50	0	30	10	60	0	20	10	70
SPA1 10^1	100	0	0	0	10	80	0	10	10	60	10	20	10	60	10	20	10	60	10	20	10	50	10	30	10	50	10	30
SPA1 10^1 + Lb21 10^6	100	0	0	0	40	50	10	0	40	50	10	0	40	50	10	0	40	50	10	0	40	50	10	0	20	70	0	10
SPA1 10^1 + Lb21 CFS	100	0	0	0	0	40	20	40	0	30	10	60	0	30	10	60	0	30	10	60	0	10	10	80	0	10	0	90
Lb21 10^6	100	0	0	0	0	100	0	0	0	100	0	0	0	100	0	0	0	100	0	0	0	80	20	0	0	80	20	0
Lb21 CFS	100	0	0	0	0	80	0	20	0	60	10	30	0	60	10	30	0	60	10	30	0	60	0	40	0	60	0	40
PBS	100	0	0	0	20	50	20	10	10	60	20	10	10	60	10	20	10	60	10	20	10	60	10	20	10	60	10	20

Yellow highlighted cells are control groups. Green highlighted cells are experimental groups which infected with the pathogen and treated with Lb21 washed cells. Blue highlighted cells are experimental groups which infected with the pathogen and treated with the CFS of Lb21.

6.5 Discussion

6.5.1 Susceptibility of *G. mellonella* Larvae to the Injected Bacterial Species

Susceptibility of *G. mellonella* larvae to the bacterial species used in this study has been evaluated by injection both of food isolated lactobacilli and pathogenic species. Results obtained from injection experiments of *Lactobacillus* species focused on finding the injected bacterial doses which showed the highest survival percentages. Whereas the goal of pathogens injection was the detection of the bacterial dose that has the ability to kill the highest number of larvae (the highest lethal dose) and the dose which killed 50% of injected larvae (LD_{50} value). Despite that, survival percentages of the larvae injected with several bacterial doses of both lactobacilli and pathogenic species were all reported. In response to the injected bacterial species, the health situation of the larvae was monitored during the incubation time, by the observation of larvae activity, melanization and survival. In a previous investigation carried out by Loh and colleagues, they checked the larvae health status depending on four main observations: activity, cocoon formation, melanization and survival. The highly active and more cocoon forming wax worms were considered as the healthier larvae (Loh *et al.* 2013).

It has been shown in this research that melanization was not always a sign of death, since some of dead larvae remained creamy coloured (the normal colour of a healthy larva), nevertheless, a number of injected larvae produced the melanin when they were still alive and became brown coloured. Melanization is a part of the infection process in insects (Aperis *et al.* 2007). The occurrence of a pathogenic infection causes the production of melanin by the wax worm as a result of the immune response against the infection, since melanin helps to trap and kill pathogens (Cerenius and Söderhäll 2004). Caterpillars showed clear signs of melanization during the progress of the pathogenic infection. When the immune system has been overwhelmed, a typical complete melanization occurs which associates with death of larvae soon after.

6.5.1.1 Survival of *G. mellonella* Injected with Food Isolated *Lactobacillus* Species

Both food isolated *Lactobacillus* species including: *Lb. delbreuckii* and *Lb. plantarum* were injected as bacterial suspensions or washed cells at the doses range of 10^1 - 10^6 CFU/larva. It was observed that the injection of the highest dose 10^6 CFU/larva had a lethal effect on the larvae and revealed either no larvae survived (0%) or low survival percentages (10% - 50%) for

all isolates of the two species, except *Lb. delbreuckii* (Lb19). Since the injection of 10^6 CFU/larva of Lb19 washed cells showed a survival percentage more than 80%, this isolate seems to be less virulent than the other isolates. This indicates that isolates which belong to the same species may vary at the strain level and could present different effect on the survival of larvae. These results were in agreement with Grounta and his colleagues who reported the virulence activity of LAB strains injected in high doses (ranging from 10^5 to 10^7 CFU/larva) on the larval survival. They found that the injection of high initial doses leads to high percentages of larval death. Thus, they stated that the infectious dose of both *Lb. pentosus* B281 and *Lb. plantarum* B282 was 5.5 log CFU/larva, while that dose was 6 log CFU/larva for *Lb. rhamnosus* GG. As a result of these findings, they decided to decrease the injected LAB dose to 10^4 CFU/larva (Grounta *et al.* 2016). Some species of *Lactobacillus* were reported for their tendency to cause sepsis in immunocompromised human patients like *Lb. rhamnosus* and *Lb. casei* (Cannon *et al.* 2005; Gouriet *et al.* 2012). Therefore, it was suggested in a recent study that more "pathogenic" *Lactobacillus* would be less appropriate as therapeutic agents in animal infection models (Stanbro *et al.* 2019).

High survival percentages of larvae were obtained when the four *Lb. delbreuckii* isolates were injected at the doses range of 10^3 - 10^5 CFU/larva, these percentages were 80% - 100% for bacterial suspensions and 60% - 100% for washed cells. In terms of the six isolates of *Lb. plantarum*, 70% - 100% of larvae survived when most bacterial suspensions were injected at the doses range of 10^3 - 10^4 CFU/larva. While injection of the same doses range of bacterial washed cells showed survival percentages ranging from 80% - 100%. In general, 10^3-10^5 CFU/larva of food isolated *Lactobacillus* presented the highest survival percentages of larvae. Similar outcomes have been detected by a previous research group who stated that the survival of larvae has not been affected by the injection of 10^4 CFU/larva (Grounta *et al.* 2016).

To conduct the co-injection experiments, two doses of both *Lb. delbreuckii* (Lb19) and *Lb. plantarum* (Lb21) washed cells were selected. These are 10^2 CFU/larva and 10^4 CFU/larva for the first species, while they were 10^3 CFU/larva and 10^4 CFU/larva for the second one. The selection of these isolates is based on two criteria. Firstly, high survival percentages (more than 80%) obtained from the injection of these doses. Secondly, the absence of significant differences between survival percentages resulted from the selected injected doses and those percentages of the PBS control group. Higher doses of bacterial washed cells were not chosen for the co-injection experiments since these doses showed either no survival or low survival percentages of larvae. Although other injected low doses presented high survival percentages and did not

show any significant differences compared to PBS group, these doses were also not selected for the co-injection suggesting that injection of the low dose of bacterial cells (10^1 CFU/larva) with the pathogen may not show any effect against the pathogenic infection. Moreover, the survival percentages obtained from the injection of these low doses were similar or close to those of the selected two doses.

In preliminary experiments, cell-free supernatants (CFSs) of *Lactobacillus* isolates were injected once. Undiluted supernatants of most of the isolates (7 from the total of 10) showed low survival percentages of larvae. Therefore, the CFSs injection was repeated in triplicate for Lb19 and Lb21 to confirm the results. Although several dilutions of supernatants were injected, it was observed that the undiluted CFS of Lb19 presented higher percentage of larvae survived than that of Lb21. Additionally, the diluted CFS 10^{-1} of Lb19 revealed the highest survival percentage (80%). Whereas the highest percentage of survival (70%) was obtained from the injection of the more diluted CFS 10^{-3} of *Lb. plantarum* (Lb21). This suggests that the larvae were able to survive in the low diluted supernatant of *Lb. delbreuckii* and could not tolerate low dilutions of *Lb. plantarum* supernatant. It was stated by Lopes and co-workers that some species of *Lactobacillus* can produce higher amounts of organic acids than other species of the same genus, and that *Lb. delbreuckii* produces relatively low concentrations of organic acids (Lopes *et al.* 2017). This may explain the ability of larvae to tolerate the low acidic CFS of *Lb. delbreuckii*, while showing low tolerance to the injected *Lb. plantarum* supernatant that may contain high amounts of produced organic acids.

6.5.1.2 Survival of *G. mellonella* Injected with Pathogenic Species

The injection of pathogenic species including: *S. aureus* (SA1), *S. pyogenes* (SPA1) and *S. dysgalactiae* subsp *equisimilis* (SDG4) was performed as two different treatments: bacterial suspensions and washed cells. The maximum incubation period for the larvae injected with bacterial suspensions was three days. Table 6.13 presents the survival percentages resulted from the injection of the highest doses of bacterial suspensions and the LD_{50} values determined for the pathogens. The highest injected dose of bacterial suspensions for both SA1 and SDG4 was 10^6 CFU/larva. Whereas, the highest injected dose of SPA1 was 10^5 CFU/larva. The virulence degree of pathogens was assessed by the comparison of the lethal effect of the maximal injected bacterial cells and LD_{50} values. It was found that SPA1 was the most virulent pathogen among the three pathogens, since the highest injected dose 10^5 CFU/larva of this pathogen killed more than 96% of infected larvae (3.3% survival) after three days (Figure 6.10A). Whereas injection

of the other two pathogens SA1 and SDG4 in the same bacterial dose (10^5 CFU/larva) resulted in higher survival rates approached 10% for both after three days of infection (Figures 6.9A and 6.11A). Although SA1 and SDG4 were both less virulent than SPA1, it was observed that SDG4 had a higher virulence than SA1. This was detected when more bacterial cells 10^6 CFU/larva of SDG4 killed all the larvae (0% survival) after only one day of infection, while the same dose of SA1 revealed 6.7% after three days. These findings were confirmed by the comparison of LD_{50} values for the three pathogens. Despite the similar LD_{50} value for both SPA1 and SDG4 which was 10^3 CFU/larva, the injection of this dose resulted in a survival rate of 43.3% for SPA1, which was slightly lower than that for SDG4 (46.7%) at the end of the incubation time. This confirmed that SPA1 was more virulent than SDG4. In comparison to SPA1 and SDG4, more bacterial cells of SA1 (10^4 CFU/larva) could kill approximately 50% of larvae (33.3%), indicating that SA1 was the least virulent pathogen.

Regarding the injection of pathogenic washed cells, the maximum incubation period was three days for SA1 and six days for the other two pathogens. Table 6.14 depicts the survival percentages resulted from the injection of the highest doses of washed cells and the LD_{50} values determined for the pathogens. The highest injected dose of both SA1 and SPA1 was 10^6 CFU/larva. While, the highest injected dose of SDG4 was 10^5 CFU/larva. It was observed that 10^6 CFU/larva of SA1 killed all the larvae after two days. Whereas when the same dose of SPA1 was injected, all the larvae died after only one day showing that SPA1 had a higher virulent activity than SA1 on the first two days of incubation. However, the injection with the lower doses of SPA1 revealed higher survival percentages (Figure 6.10B) than those obtained from the injected corresponding doses of SA1 (Figure 6.9C) on the third day of incubation. Even after the third day of incubation, the survival percentages of the larvae injected with SPA1 doses remained high (more than 60%). Therefore, the incubation time was increased into six days and the LD_{50} value of this pathogen was between 10^4 - 10^5 CFU/larva on the sixth day. It was reported by Champion and colleagues that the survival percentage of the infection model *G. mellonella* has to be checked for up to five days after the infection, to allow the determination of a maximum half lethal dose (LD_{50}) (Champion *et al.* 2016). In contrast to SA1, lower number of bacterial cells (10^3 - 10^4 CFU/larva) killed 50% of larvae after three days only. Nevertheless, injection of SPA1 in the same dose range (10^3 - 10^4 CFU/larva) revealed more than 60% of larvae survived after six days of incubation, which indicates that washed cells of SPA1 injected at these doses showed low virulence compared to SA1. It was also observed that there was no dose response when SPA1 was injected at the doses range between 10^1 - 10^3 CFU/larva, and the survival of

larvae receiving low pathogenic doses decreased. With respect to SDG4, the highest injected dose of this pathogen (10^5 CFU/larva) showed low activity for killing the larvae after three days resulting in 66.7% of larvae survived (Figure 6.11C). Although the incubation was increased into six days, the survival of larvae injected with this pathogenic dose remained high approaching 60% at the end of experiment. Furthermore, low injected pathogenic doses (10^1 - 10^4 CFU/larva) presented either slight or no lethal effect on the larvae resulting in high survival rates ranging from 90% - 100% on the sixth day of infection. Hence, LD_{50} value could not be determined for this pathogen and it was considered as the least virulent among the three pathogens.

Table 6.13 Survival percentages of *G. mellonella* injected with bacterial suspensions of S. aureus (SA1), *S. pyogenes* (SPA1) and *S. dysgalactiae* subsp equisimilis (SDG4). The table shows the highest injected doses of bacterial suspensions, LD_{50} values and the day on which these results were obtained. The incubation time of this experiment was three days.

Pathogen	The Injected Bacterial Dose (Survival Percentage %) / Incubation Day	
	The Highest Injected Dose[1]	LD_{50} Values[1]
SA1	10^6 (6.7) / 3rd day	10^4 (33.3) / 3rd day
SPA1	10^5 (3.3) / 3rd day	10^3 (43.3) / 3rd day
SDG4	10^6 (0) / 1st day	10^3 (46.7) / 3rd day

[1]Doses of bacterial suspensions and LD_{50} values are presented in CFU/larva

Table 6.14 Survival percentages of *G. mellonella* injected with bacterial washed cells of *S. aureus* (SA1), *S. pyogenes* (SPA1) and S. dysgalactiae subsp equisimilis (SDG4). The table shows the highest injected doses of washed cells, LD_{50} values and the day on which these results were obtained. The incubation time of this experiment was three days for the SA1 and six days for SPA1 and SDG4.

Pathogen	The Injected Bacterial Dose (Survival Percentage %) / Incubation Day	
	The Highest Bacterial Dose[1]	LD_{50} Values[1]
SA1	10^6 (0) / 2nd day	10^3 - 10^4 (50) / 3rd day
SPA1	10^6 (0) / 1st day	10^4 - 10^5 (33.3 - 66.7) / 6th day
SDG4	10^5 (60) / 6th day	Could not be determined[2]

[1]Doses of bacterial washed cells and LD_{50} values are presented in CFU/larva
[2]LD_{50} value of SDG4 could not be determined since all the doses of washed cells showed high survival percentages (more than 60%) after six days of incubation

The utility of *G. mellonella* as a model to investigate the virulence of *S. aureus* was first described by Peleg and colleagues, who acknowledged the efficacy of this model in simplification the *in vivo* study of the pathogen virulence. Initial experiments were performed by this research group when they injected two different strains of *S. aureus* in the larvae. The first strain A8090 was more pathogenic than the second one A8094. After 8 h of infection, it was observed that the first injected strain resulted in a higher pathogenic load in tissue compared to

that obtained from the injection of the less pathogenic strain. Cellular and humoral immune responses in *G. mellonella* are mediated by haemocytes and antimicrobial peptides, respectively. Therefore, the results obtained by this group indicated that the host immune responses may have an important influence on the killing variations caused by the pathogen inside the larvae (Peleg *et al.* 2009b).

It was found that washed cells of SPA1 and SDG4 injected at the doses of 10^1 - 10^5 CFU/larva revealed high survival percentages of larvae (more than 50%) after three-day incubation. These survival percentages remained high despite the prolonged incubation time into six days. Moreover, the LD_{50} dose of the first pathogen (SPA1) was high (10^5 CFU/larva) and difficult to be determined for the second pathogen (SDG4). After the collection of pathogens from their isolation source, they were kept in the freezer until the time of experiment. Furthermore, several rounds of sub culturing were carried out for these isolates during the research period. Thus, these findings might be due to the weakened physiological or genomic characters which resulted from the long-term storage of pathogens in freezer stocks and repeated cultivation on cultural media. Previous studies on *Bifidobacteria* spp. also reported a reduction and rapid loss of bacterial genomic regions when extensively grown in laboratory cultural media (Lee *et al.* 2008; Lee and O'Sullivan 2010).

It was reported by several studies associated with *S. pyogenes* that the bacterial pathogenicity is known to be influenced by the metabolic status of bacteria due to the regulation of expressed virulence factors (Kreikemeyer *et al.* 2003; Dmitriev and Callegari 2008). Loh and co-researchers investigated some factors affecting the virulence of Group A Streptococcus (GAS, *Streptococcus pyogenes*) towards the wax worm. The factors included in the study were the bacterial growth phase and the storage of cultures. To explore the effect of the growth phase, bacterial cultures were grown to exponential phase before the injection. A comparison was performed by the injection of fresh exponential phase cultures and fresh stationary phase cultures. Unexpectedly, fresh stationary phase cultures of GAS were found to be more virulent than fresh exponential phase ones, indicating that virulence factors expressed during the later stage of bacterial growth may be more toxic to larvae than those expressed during exponential phase. It is acknowledged that during different growth phases of GAS, several virulence factors are recognised to be differently expressed. However, numerous cell-wall associated factors tend to have a greater expression during early growth stages, whereas secreted virulence factors are more highly expressed throughout later growth stages. For instance, M-protein which is a cell wall attached protein and an important virulence factor with multiple functions including

adhesion and immune evasion. The expression of this factor is likely to be high during the exponential phase of growth to assist in host colonization. Other factors involved in tissue damage and bacterial spreading such as cysteine protease SpeB are expressed in stationary growth stage (Kreikemeyer *et al.* 2003). Thus, the increased virulence of the pathogen at the stationary phase might be because of the higher effect of the these later-expressed genes on wax worms. As secreted virulence factors are usually upregulated during the stationary phase of the bacterial growth, the wax worms were injected with filtered supernatants of fresh exponential phase and fresh stationary phase cultures to explore the toxic effects of these supernatants and to examine if these factors are related to the higher virulence of stationary phase bacteria. The survival of larvae injected with stationary phase supernatants was rapidly decreased due to the effect of the secreted virulence factors. In contrast, a complete survival was observed after the injection of exponential phase supernatants. Secreted virulence factors were also produced at the exponential phase; however, their accumulation in the supernatant was not in high levels to show a toxic effect on the larvae. Further work was conducted by the same research group to assess the influence of cultures storage on the virulence. Cultures at exponential phase were frozen and thawed at the time of inoculation to assess the storage influence on the virulence. Higher virulence was detected with fresh exponential phase cultures compared to frozen exponential phase ones, due to the active metabolic state of fresh cultures prior to inoculation (Loh *et al.* 2013). While this investigation clarified the toxic effect of virulence factors during different phases of bacterial culture growth, it could also explain the difference between the virulence effect of the bacterial suspensions and washed cells of *S. pyogenes* (SPA1) (Figure 6.10A and B) and *S. dysgalactiae* subsp *equisimilis* (SDG4) (Figure 6.11A and C) involved in the present study. Bacterial suspensions of these two pathogens had more lethal effect than bacterial washed cells as high survival was shown after the injection of washed cells. Since the bacterial suspension contains both bacterial cells and the secreted virulence factors produced by the bacteria such as toxins, thus it is expected to be more virulent than the washed cells. Furthermore, the low toxic effect of washed cells was due to the influence of the virulence factors associated with bacterial cell wall but not the secreted compounds that play an important role in the toxicity effect of pathogens in the larvae. Cell wall associated virulence factors such as M-protein are responsible for the first stages of the pathogenicity which are the colonization and immune response stimulation. To some extent, the colonization stage is avoided by the direct injection into the hemocoel (Loh *et al.* 2013).

In experimental steps conducted for the preparation of the injected washed cells of SDG4, it was found that this pathogen showed a weak growth rate during the first day of *in vitro* incubation. The impaired bacterial growth may also contribute to the obtained low virulence of SDG4 inside the larvae and the decreased lethal activity of injected doses. These results were compatible with the findings of a study carried out by Peleg and co-workers, who stated that slower growth of *S. aureus* could be one of the reasons of the attenuated virulence (Peleg *et al.* 2009b).

6.5.2 Challenge of *G. mellonella* with *Lactobacillus* and Pathogenic Species

6.5.2.1 The Injected Doses Range of Pathogenic Species with the Maximum Lethal Effect

As shown in the results, each co-injection experiment was designed to inject one of the three pathogenic species with one of the two *Lactobacillus* isolates. The first comparison in the individual co-injection experiment was performed between the survival percentages of larvae injected with each pathogenic dose and that of PBS control group. The purpose of this comparison was to determine the lethal doses range of injected pathogens which highly affected the survival of larvae resulting in low number of larvae survived in comparison to the PBS injected group. In all experiments, PBS groups showed a range of survival percentages between 80% - 100% on the sixth day of incubation. Comparison of this percentages range with the survival percentages obtained from the injection of each pathogenic dose, allowed the recognition of the pathogenic doses range with the maximum lethal effect on the larvae. *S. aureus* (SA1) was extremely lethal in the injected doses range of 10^3 - 10^6 CFU/larva which revealed survival percentages ranging from 0% - 40% at the end of incubation days. Whereas *S. pyogenes* (SPA1) showed a high virulence when injected at the doses range of 10^5 - 10^6 CFU/larva and the survival of larvae approached to 0% - 45%. In terms of *S. dysgalactiae* subsp *equisimilis* (SDG4), the injected doses range of 10^3 - 10^4 CFU/larva had a high lethal effect on the larvae resulted in 30% - 40% of larvae survived. Despite the large difference in survival percentages between these pathogenic doses and PBS group, the statistical analysis showed significant differences for some comparisons, but it was trending towards significance for others. All these doses were recognised as the injected doses with the maximum lethal effect, due to their high impact on the larval survival resulting in all larvae died or lower than 50% of larvae survived after six days of infection. Injection of low pathogenic doses revealed high survival of larvae and no significant differences compared to PBS. Therefore, these doses were considered as less lethal doses of the pathogenic species.

6.5.2.2 The Lethal Effect of Injected Pathogenic and *Lactobacillus* Doses on the Survival of Larvae

The second comparison of survival percentages was carried out between the larvae injected with each pathogenic dose and the groups receiving each of the two *Lactobacillus* doses. This comparison aimed to evaluate the virulence of all injected bacterial doses and to find out the pathogenic doses range which had more lethal effect on the larvae than the injected doses of *Lactobacillus*. Although the two selected doses of *Lactobacillus* resulted in high survival percentages, lower number of injected bacterial cells revealed higher percentages of survival. In the co-injection experiments associated to *Lb. delbreuckii* (Lb19), injection of the first dose 10^2 CFU/larva showed 80% - 90% of larvae survived. Whereas the second injected dose 10^4 CFU/larva revealed survival percentages of 65% - 90%. Regarding the experiments related to *Lb. plantarum* (Lb21), the two injected doses 10^3 CFU/larva and 10^4 CFU/larva showed survival rates of 85% for the first dose and 65% - 70% for the second one.

All the injected doses of *S. aureus* (SA1) ranging from 10^1 - 10^6 CFU/larva showed 0% - 90% of larvae survived which were lower than those obtained from the injection of Lb19 two doses. Therefore, all pathogenic doses of SA1 were more lethal than each of the two injected doses of Lb19. However, the lowest pathogenic dose that contained 10^1 CFU/larva presented similar percentage of survival to Lb19 doses. The same pathogenic doses (10^1 - 10^6 CFU/larva) revealed survival percentages of 0% - 80% in the co-injection experiment performed with Lb21. These pathogenic doses also showed higher lethal effect than the two injected doses of Lb21. Nevertheless, the high dose of Lb21 (10^4 CFU/larva) was slightly more lethal than 10^1 CFU/larva of SA1.

With respect to *S. pyogenes* (SPA1), the injected doses range of 10^1 - 10^6 CFU/larva had higher lethal activity than Lb19 two doses. Nonetheless, the high injected dose of Lb19 (10^4 CFU/larva) and the lowest pathogenic dose (10^1 CFU/larva) both showed similar percentage of survival. The same pathogenic doses range showed survival rates of 0% - 80% and was more lethal than 10^3 CFU/larva of Lb21. However, only the high doses of SPA1 including 10^5 - 10^6 CFU/larva that presented 0% - 30% of larvae survived, had a higher lethal action than 10^4 CFU/larva of Lb21.

In terms of the third pathogen *S. dysgalactiae* subsp *equisimilis* (SDG4), it was injected at the doses range of 10^1 - 10^4 CFU/larva with Lb19. The pathogenic injected doses of 10^2 - 10^4 CFU/larva were more lethal than 10^2 CFU/larva of Lb19, as they showed lower survival

percentages (30% - 65%) compared to that obtained from the injection of L19 dose. The pathogenic doses range of 10^3 - 10^4 CFU/larva had more lethal effect than 10^4 CFU/larva of Lb19. Furthermore, both of Lb19 two dose were more lethal than the lowest pathogenic dose contained 10^1 CFU/larva. In general, all the injected pathogenic doses had higher lethal activity than the doses of *Lactobacillus* with an exception of the lowest dose of pathogens which either revealed lower or similar lethal effect compared to that of *Lactobacillus* doses.

6.5.2.3 The Co-injected Doses Range with the High Lethal Effect

In each co-injection experiment, the third comparison of survival percentages was performed between the PBS control group and the larvae co-injected with both of the pathogen and *Lactobacillus*. The purpose of this comparison was to determine the lethal co-injected dose that showed a high influence on the survival of larvae resulting in less than 50% of larvae survived in comparison to the PBS injected group. Furthermore, the highly lethal co-injected dose was recognised when it revealed a difference in survival percentages equal or more than 50% compared to the PBS group.

Co-injection of *S. aureus* (SA1) at the doses range of 10^4 - 10^6 CFU/larva with any of *Lb. delbreuckii* (Lb19) doses revealed survival percentages of 0% - 45%. Furthermore, when 10^5 - 10^6 CFU/larva of this pathogen was co-injected with any of *Lb. plantarum* (Lb21) doses, only 0% - 20% of larvae survived. The obtained survival rates were an indication that these co-injected doses highly affected the larval survival. Regarding the co-injection experiments of *S. pyogenes* (SPA1), the survival of larvae highly influenced by the injection of the pathogenic dose 10^6 CFU/larva with any of Lb19 doses resulting in all larvae died. Additionally, low survival rates were obtained from the injection of doses contained 10^3 - 10^6 CFU/larva of SPA1 with 10^3 CFU/larva of Lb21 (0% - 40%), and doses contained 10^5 - 10^6 CFU/larva of the pathogen with 10^4 CFU/larva of Lb21 (0% - 30%). With respect to *S. dysgalactiae* subsp *equisimilis* (SDG4), injection of the highest pathogenic dose 10^4 CFU/larva with 10^2 CFU/larva of Lb19 showed only 15% of larvae survived. Moreover, all the pathogenic doses (10^1 - 10^4 CFU/larva) co-injected with 10^4 CFU/larva of Lb19 revealed survival percentages of 5% - 35%. In all experiments, the range of survival percentages obtained from the injection of PBS was 80% - 100%. The comparison of the PBS injected larvae with the groups receiving the co-injected doses mentioned above, showed a high difference in survival percentages. Thus, these co-injected doses were considered as the doses which had the maximum virulence on the survival of larvae.

6.5.2.4 The Co-injected Doses Range with the Low Lethal Effect

In the fourth comparison of survival percentages, the group co-injected with the pathogen and one of the two *Lactobacillus* doses was compared to the larvae injected with the same dose of *Lactobacillus*. This comparison was conducted to find out the co-injected dose with the low lethal effect on the larval survival. This co-injected dose was the one which showed a close survival percentage to that obtained from the *Lactobacillus* dose injected alone (the same dose injected with the pathogen). The small difference in survival percentages (less than 50%) was an indication of the less lethal co-injected dose. Moreover, the survival percentage resulted from this co-injected dose was more than 50%.

As the injection of the two doses of *Lactobacillus* species showed high survival percentages, these doses were selected for the challenge experiments with the pathogens. In all co-injection experiments, the survival percentages resulted from the injection of Lb19 doses were at the range of 80% - 90% for 10^2 CFU/larva and 65% - 90% for 10^4 CFU/larva. Whereas the injection of Lb21 doses revealed a survival percentage of 85% for 10^3 CFU/larva and the range of survival percentages was 65% - 70% for 10^4 CFU/larva. The challenge experiments of *S. aureus* (SA1) showed that co-injection of this pathogen at a specific doses range with any of *Lactobacillus* doses, presented high percentages of survival (more than 50%) which were slightly different to those obtained from *Lactobacillus* dose injected alone. These co-injected doses including: 10^1 - 10^3 CFU/larva of SA1 with any of Lb19 doses, 10^1 - 10^4 CFU/larva of this pathogen with 10^3 CFU/larva of Lb21 and 10^1 - 10^2 CFU/larva of the pathogen with 10^4 CFU/larva of Lb21. These co-injected doses were recognised as the less lethal doses that slightly affected the survival of larvae. With respect to the co-injection experiments of *S. pyogenes* (SPA1), all the pathogenic doses lower than 10^6 CFU/larva (10^1 - 10^5 CFU/larva) injected with any of Lb19 doses, 10^1 - 10^2 CFU/larva of the pathogen with 10^3 CFU/larva of Lb21 and 10^1 - 10^4 CFU/larva of SPA1 with 10^4 CFU/larva of Lb21 were all distinguished as the doses with the low lethal activity on the larval survival. In terms of the third pathogen *S. dysgalactiae* subsp *equisimilis* (SDG4), the less lethal co-injected dose was the one contained 10^1 - 10^3 CFU/larva of the pathogen and 10^2 CFU/larva of Lb19. The high survival percentages obtained from these co-injected doses of all three pathogens were at the range of 50% - 95%, while the overall differences range in survival percentages between these co-injected doses and *Lactobacillus* doses was 0% - 35%.

Although few studies were assessed the efficacy of LAB administration against some pathogens in *G. mellonella*, to the best of our knowledge there are no investigations in the literature that were carried out the comparisons between groups of larvae co-injected with different doses of *Lactobacillus* species and pathogens. Therefore, no direct comparison can be discussed for the results of some comparisons performed in the present work with previous studies since the scope between the experiments are different.

6.5.2.5 The Therapeutic Potency of *Lactobacillus* Injected Doses Against Pathogenic Infections

The fifth comparison in each co-injection experiment was conducted between the survival percentages of treated larvae (larvae injected with *Lactobacillus* after the infection of the pathogen) and those of infected larvae (larvae injected with the pathogen alone). The goal of this comparison was to determine the therapeutic injected dose of *Lactobacillus* species which showed increased survival percentages of treated larvae in comparison with non-treated larvae. As mentioned in the results, groups of larvae were infected with several doses of each pathogen and treated with two doses of both *Lb. delbreuckii* (Lb19) and *Lb. plantarum* (Lb21).

In each co-injection experiment, the highest injected pathogenic dose was 10^6 CFU/larva for both *S. aureus* (SA1) and *S. pyogenes* (SPA1), while it was 10^4 CFU/larva for *S. dysgalactiae* subsp *equisimilis* (SDG4). No increase in survival percentages was found for the larvae challenged with the highest doses of pathogens and any of *Lactobacillus* doses compared to the larvae infected with these pathogenic doses. Although the larvae injected with the selected doses of *Lactobacillus* species remained alive with high survival rates after six days, it appeared that the larvae were not able to tolerate the high number of injected bacterial cells when any dose of *Lactobacillus* injected with the high pathogenic dose.

Although no statistical significance observed for both *Lactobacillus* species evaluated, treatment with these two species increased the survival of larvae infected with specific pathogenic doses. Regarding *Lb. delbreuckii* (Lb19), treatment with 10^2 CFU/larva of this species was effective against infections caused by the three pathogens. Groups of larvae infected with 10^2 - 10^5 CFU/larva of SA1 revealed survival rates ranging from 20% - 65%, this percentages range increased into 35% - 90% when these groups treated with 10^2 CFU/larva of Lb19 and the increase rate of survival was 15% - 25%. While the larvae infected with the lowest pathogenic dose 10^1 CFU/larva showed similar survival percentage (90%) for both untreated and treated groups. Furthermore, infection caused by 10^1 - 10^5 CFU/larva of SPA1 resulted in 45% -

85% of larvae survived, which increased into 60% - 95% for the groups treated with the same dose of Lb19 (10^2 CFU/larva) showing an increase range of 10% - 20%. Moreover, the survival of larvae previously infected with 10^2 - 10^3 CFU/larva of SDG4 and treated with this dose of Lb19, showed higher survival percentages ranging from 60% - 90% compared to the percentages of larvae infected with pathogen alone which were 40% - 65%. The survival of larvae infected with the lowest dose of this pathogen was 95% and remained the same after the treatment. In terms of the second injected dose 10^4 CFU/larva of Lb19, it had a therapeutic potency against infections caused by SA1 and SPA1. However, 10^4 CFU/larva of Lb19 was less effective than 10^2 CFU/larva against SA1 infection, as the first dose (the high dose) showed a therapeutic effect on the larvae infected with 10^2 - 10^4 CFU/larva of the pathogen, while the second dose (the low dose) was effective against a slightly wider range of pathogenic doses (10^2 - 10^5 CFU/larva). Larvae infected with 10^2 - 10^4 CFU/larva of SA1 showed survival rates of 20% - 65%, which increased into 45% - 85% after the treatment with 10^4 CFU/larva of Lb19. This dose of Lb19 also showed an efficiency for the treatment of SPA1 infection. It was observed that the larvae infected with the pathogenic doses ranging from 10^1 - 10^5 CFU/larva presented 45% - 85% of larvae survived, this range of survival increased into 60% - 95% for the larvae treated with 10^4 CFU/larva of Lb19 with an increase range of 10% - 25%. No therapeutic effect was found for 10^4 CFU/larva of Lb19 against the infection caused by SDG4.

With respect to *Lb. plantarum* (Lb21), the injected dose 10^3 CFU/larva increased the survival of larvae infected with a broad range of SA1 doses 10^1 - 10^5 CFU/larva. The survival percentages range of infected larvae was 15% - 80%, which increased into 20% - 85% for the treated group of larvae, resulting in an increase in survival percentages with a range of 5% - 55%. In contrast to 10^3 CFU/larva of Lb21, the bacterial dose10^4 CFU/larva was less effective against SA1 infection, as the latter dose showed an increase in the survival of groups infected with a small range of pathogenic doses (10^3 - 10^4 CFU/larva) and the increase range was 20% - 30%. No remarkable therapeutic effect was observed for the two Lb21 doses against SPA1 infection, as the injection of these doses did not show an increase in survival percentages for the larvae previously infected with any of the pathogenic doses. Nevertheless, both of Lb21 doses were able to slightly decrease the lethal activity of low pathogenic doses, including 10^2 CFU/larva and 10^1 CFU/larva, resulting in small increase (5% - 10%) in survival percentages of larvae infected with these doses.

Overall, the injected dose of *Lactobacillus* which contained low number of bacterial cells had more therapeutic effectiveness than the high dose against the pathogenic infections. Moreover, the observed increase in survival rates of larvae infected with target pathogens and treated with *Lactobacillus* species further emphasise that live cells of selected bacterial species can be used as promising therapeutic agents against skin infections. The effect of lactic acid bacteria with probiotic potential was studied by Grounta and co-workers against *Staphylococcus aureus* and *Listeria monocytogenes* infections. LAB strains involved in this investigation were *Lactobacillus rhamnosus* GG (inhabitant of human intestinal tract), *Lactobacillus pentosus* B281 and *Lactobacillus plantarum* B282 (isolated from table olive fermentation). These strains were examined as protective agents in the context of prophylaxis and as therapeutic agents in the context of therapy. Within the context of prophylaxis, live or heat-killed cells of each LAB strain were injected at the dose of 10^4 CFU/larva at 6 h or 24 h prior to pathogenic infection. Within the context of therapy, 1/10 (10^{-1}) cell free supernatants (CFSs) of LAB cultures were injected in the larvae previously infected with the pathogen. In the prophylaxis treatment, it was declared by the research group that the survival of infected larvae greatly influenced by the time of challenge period prior to infection (6 h or 24 h) and the type of LAB cells (live or heat-killed cells). In general, it was observed that the treatment with both types of LAB cells prior to infection revealed slower killing of infected larvae, indicating that both cell types were effective in the protection against the infections caused by both pathogens. However, the highest survival percentages of larvae were found for the treatment with heat-killed cells. For example, attenuation of *S. aureus* infection was detected when the larvae were administered with live cells of *Lb. plantarum* B282 strain under both challenge periods. Whereas heat-killed cells of all LAB strains attenuated the infection under 24 h challenge period. In terms of the therapeutic effect against both pathogenic infections, no extended survival was observed in any of the groups treated with CFSs compared to the control groups of larvae (Grounta *et al.* 2016). According to these results, live cells of *Lb. plantarum* B282 were considered as efficient protective agents against *S. aureus* infection, while no therapeutic effect was found for the CFS of this species against the infection. In contrast, the bacterial cells of *Lb. plantarum* (Lb21) (involved in this study) were used as therapeutic agents and showed an increase in survival rates of larvae previously infected with *S. aureus* (SA1). Both of *Lb. plantarum* B282 and Lb21 were isolated from fermented olives.

In a previous study conducted by Santos and colleagues (Santos *et al.* 2019), the bacterial suspensions containing 10^6 cells/larva of *Lactobacillus paracasei*, *Lactobacillus fermentum* and *Lactobacillus rhamnosus*, were assessed for their prophylactic and therapeutic effects against the infections of *Candida albicans* and non–*albicans* species in *G. mellonella* model. It was found that *Lb. rhamnosus* demonstrated a therapeutic efficacy against *C. albicans*, *C. krusei* and *C. tropicalis* infections. Whereas the other two *Lactobacillus* species increased the survival of larvae infected with non–*albicans* species. However, the prophylactic treatment of *Lactobacillus* species showed greater advantages during *Candida* spp. infection, in comparison to the therapeutic treatment. Therefore, different *Lactobacillus* species were considered as potent prophylactic agents of infections caused by *Candida* species (Santos *et al.* 2019). Another research group also investigated the effect of live cells and supernatant filtrates of *Lb. acidophilus* ATCC 4356 at 1 h pre- and post-infection with *C. albicans* in *G. mellonella* larvae. A significant survival prolongation was observed after the treatment with either LAB culture or filtrate as prophylactic and therapeutic agents (Vilela *et al.* 2015).

Despite of large differences between survival percentages of two comparison groups of larvae in performed co-injection experiments of the present study, it was observed that statistical differences were either not significant for some comparisons or trending towards significance for others. Due to limitation of the study size, each challenge experiment was carried out in duplicate. Although the total number of injected larvae was 420 larvae per experiment, it was recommended that increasing the replicates could improve the statistical power. To confirm that, simulation has been done for a group of data pretending that the experiment was performed in triplicate not in duplicate. It was found that the statistical difference of compared groups which were not significant become statistically significant after the simulation. However, the noticeable differences in larval survival between the treated and untreated groups allowed the suggestion of the therapeutic activity of the tested *Lactobacillus* species against skin infections.

Various *Lactobacillus* species have an antagonistic effect on pathogenic bacteria. Nevertheless, there are few studies emphasizing on the activity of *Lactobacillus* species isolated from fermented food against skin pathogens in *G. mellonella* model. In this research, the therapeutic use of *Lb. delbrueckii* and *Lb. plantarum* isolated form yogurt and olive products, respectively, presented an effective therapeutic activity against *Staphylococcus* and *Streptococcus* skin infections in *G. mellonella*. Thus, these *Lactobacillus* isolates could be used as a promising alternative treatment of skin infections caused by these pathogens. However, further investigations on the treatment and prophylaxis effect of these two *Lactobacillus* species

against skin infections will be required prior to their practical use in the context of current treatments.

6.5.3 Topical Application of Pathogens and *Lactobacillus*

The effectiveness of the topical application of probiotics in the wound healing process was identified by some previous investigators. For instance, in a limited clinical study undertaken by Peral and co-researchers, patients with second and third-degree burn wounds were treated with *Lactobacillus plantarum*. The findings of this clinical investigation showed a decline in the bacterial load of the wounds and an acceleration in the wound recovery rate which may be resulted from the local treatment with this species (Peral *et al.* 2009). Another study stated that *Lb. plantarum* has the ability to interfere *in vitro* with the virulence factors and biofilm formation of *P. aeruginosa*, it could also stimulate the immune response and tissue repair in an experimental burn murine model (Valdez *et al.* 2005). It was reported by Gan and colleagues that *Lactobacillus fermentum* RC-14 and its produced compounds could inhibit *S. aureus* infections after the surgical implantation. *Lb. plantarum* may have a similar activity in surgical implant infections, since its potency was proved in reducing the number of *S. aureus* and other microorganisms in burn infections (Gan *et al.* 2002). Depending on these investigations which confirmed the usefulness of *Lb. plantarum* in skin infections, this species was used in the present experiment to evaluate its activity against *S. pyogenes* skin infection. Furthermore, no studies demonstrated any possible virulence activity *Lb. plantarum* in experimental models (Valdez *et al.* 2005). It was also mentioned by Peral and colleagues that the preparation of *Lb. plantarum* cultures could be performed in any laboratory with minimum requirements, and the treatment using this species is economically effective and easy to apply (Peral *et al.* 2009).

In this study, the trial performed to infect the larvae by the topical application of pathogenic inoculums directly to the larval dorsum was not a suitable technique for infection. This was due to the pathogen failure to enter the intact layer covering the larval body and create an infection. Thus, the infection was achieved by scratching the area before the inoculation to allow the pathogen to enter through the damaged layer and cause pathogenesis. As known, intact skin of human and animals is the physical barrier that provides some protection against different pathogens which cannot enter into the body in normal conditions. However, these pathogens usually enter the body through skin breaks generated by accidents, burns or injuries. In contrast, insects have the cuticle as an external layer that covers the body and protects from infections. Lecuona and colleagues mentioned that a waxy layer covers the outer layer of cuticle (the

epicuticle). This waxy layer consists of fatty acids, sterols and lipids which may show anti-microbial features (Lecuona *et al.* 1997). The chemical structure of the cuticle is chitin fibrils embedded in a protein matrix. Microbial pathogens are usually prevented from entering the insect's body cavity by the intact cuticle layer. However, the opportunity of infection increases when this layer is fractured by injury or degradation (Teetor-Barsch and Roberts 1983). This confirms the results obtained from the present study regarding the failure to cause a pathogenic infection without making an injury in the larval body. Kavanagh and Reeves stated that the lesion may be plugged and subsequently restored to recover the cuticle structure and function after damage. As a result of cuticle injury, humoral immune response is activated and antibacterial compounds are produced such as attacins and cecropins (Kavanagh and Reeves 2004).

The findings of the present experiment showed that the topical application of 10^6 CFU/larva of *Lb. plantarum* (Lb21) washed cells to the larval dorsum infected with *S. pyogenes* (SPA1), revealed lower death percentages than those of the control groups infected with all pathogenic doses (10^1 - 10^4 CFU/larva). Moreover, larvae treated with Lb21 bacterial cells presented lower percentages of death than the groups treated with the cell free supernatant of this bacteria. The application of the CFS to the area infected with the highest dose of SPA1 (10^4 CFU/larva) presented less death than the control group (larvae infected with this pathogenic dose). Whereas, death percentages of the groups infected with the rest of pathogenic doses (10^1 - 10^3 CFU/larva) and treated with the CFS were either similar to or higher than those of the control groups indicating that the CFS of Lb21 was not as much effective as the bacterial cells. It has been suggested that lactic acid-producing bacteria are successful candidates to stimulate the healing of the wound infection by several mechanisms like secretion of antimicrobial compounds like lactic acid and bacteriocins, decrease of the pH, and suppression of pathogenic virulence factors (Sekhar *et al.* 2014). Another study conducted by Valdez and co-workers who reported that acidic supernatant of *Lb. plantarum* cultivated on MRS, played an important role in the interference with the virulence factors of *P. aeruginosa* in burn infections (Valdez *et al.* 2005). Also, the acidic pH could assist in the activation of the immune response and in tissue repair (Lardner 2001). The topical application of *Lactobacillus* supernatant was proved to be safe on intact skin. No inflammatory effects were observed after the application of the pharmaceutical formulations contained *Lb. plantarum* ATCC 10241 supernatant to healthy volunteers (Sesto Cabral *et al.* 2015).

Although the topical application of bacterial cells on the wax worm was an informative infection technique, some steps should be performed in this experiment for more investigation

of *Lactobacillus* therapeutic ability against the pathogenic infection. Firstly, the washed cells solution of *Lactobacillus* was applied once on the infected area after the pathogen inoculation. However, a new washed cells solution should be prepared every day and applied once a day over the experiment period. It was found by Blanchet-Réthoré and co-researchers that patients with atopic dermatitis (AD) who applied heat-treated (HT) *Lactobacillus johnsonii* NCC 533 twice-daily in a moisturizer lotion showed a decrease in *S. aureus* load, indicating that the daily application could maintain the beneficial effects of topical HT bacteria and their influence on the binding ability of the pathogen (Blanchet-Réthoré *et al.* 2017). Secondly, the injury development was monitored for six days only, while the follow up period must be at least ten days to determine the utility of the applied *Lactobacillus* solution against the infection. In addition to that, despite the importance of the macroscopic examination which was used to evaluate the injured area in this study, it is necessary to investigate the microscopic and cultural features of this area as well. This investigation provides information about the presence and viability of the inoculated bacterial species in the infected area. Furthermore, it reveals the existence of other opportunistic bacterial species that may infect the injury surface of the larvae. It was acknowledged by Stanbro and co-researchers that *Lactobacillus* cultures incubated for several days were more effective as treatments, since older cultures allow the maximum accumulation of antimicrobial compounds and increased therapeutic potential. Moreover, the careful selection of *Lactobacillus* species must be taken into consideration regarding the produced lactic acid and the ability to cause sepsis in immunocompromised patients. These are considered as important therapeutic indicators (Stanbro *et al.* 2019).

It has been proposed by Ericosson and co-investigators that the accurate selection of the most instructive species for an animal model is very necessary. However, it also displays an exclusive challenge for researchers. There are several aspects associated with the selection of the suitable animal model in experiments. In addition to the economic practicality of the chosen model and the utilization of a specific species by previous investigations, scientists have to take into consideration the accessible molecular and imaging methods for a species. Also, the different biological features of that species (Ericsson *et al.* 2013). Regarding the invertebrate model *G. mellonella*, it has many advantages compared to other vertebrate models like the low cost and ease of use. Also, its ability to survive at the human body temperature of 37ºC, allows the expression of specific virulence factors of the microbes (Konkel and Tilly 2000).

Despite all advantages which can result from animal biomedical research, negative or unpredictable findings were detected by several studies. It was stated by Kalueff and colleagues

that animal models are infrequently not able to represent the complication of human behavioural interferences, and have restricted capacity for the detection of some impacts (Kalueff *et al.* 2007). In terms of *G. mellonella*, there are some drawbacks related to the use of this model such as the absence of complete genome sequence and the necessity for standardization across different sources and laboratories (Junqueira 2012; Nathan 2014). However, these disadvantages are likely to be improved in the future.

To our best of knowledge, the topical application of pathogens and lactobacilli on *G. mellonella* body surface has not been reported in the literature, since most studies either used the injection as a classical infection method in the larvae or included other animal models such as mice to investigate and treat wounds and burns infections. Although the topical application of bacterial species was carried out in the present study, there is an importance for a more in-depth investigation of the effects of experimental design before we can properly prove the wax worm for studying the topical infection of skin pathogens.

6.6 Conclusions

1- Survival curves of *G. mellonella* larvae associated with the injected doses of *Lactobacillus* species, showed different percentages of survival. However, the high bacterial doses resulted in the greater number of deaths in larvae groups, regardless the type of injected solution (bacterial suspensions or washed cells).

2- The highest survival percentages of larvae were obtained from the injection of *Lactobacillus* washed cells at the doses range of 10^3 - 10^5 CFU/larva. Similar or slightly lower doses of *Lactobacillus* bacterial suspensions revealed the highest number of larvae survived.

3- Injection of the less diluted cell free supernatant of *Lb. delbrueckii* presented the highest percentages of survival, whereas more diluted CFS of *Lb. plantarum* showed the highest survival rates.

4- The washed cells of injected pathogenic species showed lower lethal effect on the larvae than the pathogenic bacterial suspensions. Furthermore, the lethal dose which was required to kill half of the injected larvae (LD_{50}), was higher for pathogenic washed cells than that of the bacterial suspensions and was only determined after increasing the incubation time for larvae

injected with *S. pyogenes* washed cells. Whereas it could not be determined for the injected washed cells of *S. dysgalactiae* subsp *equisimilis.*

5- Both of *Lb. delbrueckii* and *Lb. plantarum* isolated from fermented food products showed a remarkable therapeutic effect against pathogenic infections in *G. mellonella* larvae. The extent to which each species exerts this feature greatly depends on the injected dose.

6- Injection of *Lb. delbrueckii* at the doses of 10^2 CFU/larva and 10^4 CFU/larva had the potential to decrease the infections caused by the two pathogens *S. aureus* and *S. pyogenes*. However, the low injected dose of this species was more effective than the high dose in reducing the infection of *S. dysgalactiae* subsp *equisimilis* in the larvae.

7- Injection of *Lb. plantarum* at the doses of 10^3 CFU/larva and 10^4 CFU/larva was successful in reducing the infection of *S. aureus* and the more effective dose was the low dose. No therapeutic activity was detected for the two doses against *S. pyogenes* infection, only a slight decrease in the lethal effect of low injected doses of this pathogen.

8- The topical application of skin pathogens to the *G. mellonella* larvae provided an important knowledge about this novel technique of infection using this model.

9- The topical treatment of skin infection caused by *S. pyogenes* with *Lb. plantarum* washed cells and undiluted cell free supernatant (CFS) showed a decline in death percentages of the treated larvae compared to the untreated groups. However, the treatment with *Lb. plantarum* washed cells was more efficient than the treatment with the CFS.

6.7 References

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Chapter Seven

General Discussion and Conclusions

7 General Discussion and Conclusions

7.1 Introduction

Investigations have been conducted in this thesis regarding the antibacterial potency of LAB against the major pathogens causing skin infections, explaining the value of fermented dairy and non-dairy food products as isolation sources of LAB with multiple probiotic characteristics. This study has further explored the genomic content of each bacterial species including LAB and pathogenic isolates. Furthermore, it has detected both the inhibitory activity *in vitro* and therapeutic effectiveness *in vivo* of food-isolated lactobacilli against clinical skin pathogens. This research has generated interesting results associated with the use of the Greater wax moth larvae *Galleria mellonella* as an *in vivo* model of infection and, for the first time, carried out multiple comparisons among different doses of food lactobacilli and skin pathogens. In addition to the classical injection method used to infect the wax worm in this study, a novel infection technique was also performed. This strategy, which has never been reported by previous studies, was the topical application of both pathogens and food lactobacilli on the dorsum of larvae. Consequently, the recent research has proved the therapeutic effect of particular doses of two *Lactobacillus* species against the pathogenic infection by using the injection method. Moreover, the second novel technique of infection has also revealed a promising activity of the topically applied *Lactobacillus* in increasing the survival of infected larvae, therefore paving the way for future research within this field.

Several questions were established prior to commencement of this research. Four experimental chapters were included in the thesis. Each chapter has delivered the resolution of the individual research question and clarified the impact of some experimental factors which believed to be affecting the obtained findings. In Chapter 3, isolation of several *Lactobacillus* species was conducted from three fermented food products including, yogurt and two types of olives. Identification of food isolates, as well as pathogenic species was undertaken by using 16S rRNA gene sequencing. In addition, whole genome sequencing was performed for all bacterial isolates and genomic analysis was carried out for the draft genomes, investigating the genes encoding for antibacterial compounds in *Lactobacillus* genomes, and detecting the virulence factors encoding gens in each pathogenic genome. Chapter 4 investigated the bacterial community of food samples used in Chapter 3 using NGS technique. The impact of two cultural

media and incubation conditions (aerobic and anaerobic) on the bacterial biodiversity was examined. Comparison between the culture-independent (NGS) and cultural dependent methods was also assessed in this chapter. Chapter 5 focused on the detection of the antibacterial effect of *Lactobacillus* species *in vitro* including, food isolates and type strains against the pathogens using overlay method. Exploration of the influence of experimental factors on the inhibitory activity was conducted such as, the source of bacterial isolation, the pH of cultural medium and incubation conditions. This chapter also investigated the bioactive substances associated with the antibacterial potency of *Lactobacillus* species. The final experimental chapter, Chapter 6, assessed the susceptibility of the *Galleria mellonella* larvae model, to various injected treatments of food-based *Lactobacillus* and recognised the injected dose with the highest survival percentages obtained. Investigation of the larvae susceptibility to different injected treatments of skin pathogens, was also performed to distinguish the injected bacterial dose with the maximum lethal effect. To evaluate the therapeutic effectiveness of two food *Lactobacillus* isolates against bacterial infections, co-injection of several doses of pathogenic and food isolates was carried out in the model *in vivo*. Finally, this chapter appraised the efficacy of food isolated *Lactobacillus* as a topical treatment of skin infections created on the body surface of the wax worm *Galleria mellonella* larvae, exploring the potency of this novel infection technique.

A couple of objectives have been listed in Chapter 1 (Introduction) to fulfil the main aim of this project. In this discussion, it will be stated to what degree have these goals been achieved. The main discussion parts and conclusions of each chapter will be reaffirmed. In addition, this chapter will discuss the involvements of this research to the widespread scientific society and probiotics biotechnology research, in particular. Moreover, the key points for future investigations will be featured and their contribution in improving the experimental conditions and exploring of further knowledge related to the probiotic applications in other models *in vivo*.

Attention will be also drawn to the importance of using food-based probiotic bacteria freshly acquired from available food products and prepared as ready formulations rather than those reserved in different culture collections.

7.2 Isolation of Lactic Acid Bacteria from Fermented Foods and Genomic Analysis of Bacterial Species (Chapter 3)

Fermented dairy products were acknowledged by numerous previous investigations as important isolation sources of LAB as these bacteria exist naturally as natural microbiota within

these products such as yogurt (Coeuret *et al.* 2003; Abed 2013). Non-dairy products such as olives were also recognised as rich sources for a variety of LAB species (Hurtado *et al.* 2012). The aim of this chapter was to identify the species of LAB which inhabit the fermented food products including, yogurt and olive types (Zer and Altunsa). The identification of food isolates and pathogenic species was conducted by 16S rRNA gene sequencing. Moreover, the genomic DNAs extracted from food-born species and pathogens were subjected to the WGS technique and analysis of the genomic data existed within the provided draft genomes. The goal of the conducted genomic analysis of food-based lactobacilli was to find out the genes responsible for the production of antibacterial substances that may play a role in the growth inhibition of pathogens. Whereas the analysis of pathogenic genomes has targeted the genes encoding for bacterial enzymes and toxins associated with the virulence of these pathogens.

The results of this study showed that *Lactobacillus* species, which have been isolated from yogurt sample were all identified as *Lb. delbrueckii*, while the rest of species isolated from table olive products were classified as *Lb. plantarum*. Depending on these findings, it can be stated with confidence, that fermented food products involved in this research were rich resources of LAB. It has been stated by several previous studies that *Lb. plantarum* was the predominant species contributed in the fermentation of table olives (De Bellis *et al.* 2010; Abriouel *et al.* 2011; Doulgeraki *et al.* 2013). *Lb. delbrueckii* is one of the essential bacterial species for the production of yogurt (Coeuret *et al.* 2003). The classification of the pathogenic and food-isolated species by 16S rRNA gene sequencing has enabled the next step in this research to take place which was the analysis of WGS data.

The results of WGS analysis revealed that *Lactobacillus* genomes have genes encoding for antibacterial compounds such as, fatty acids which were found in *Lb. delbrueckii* genomes and other genes related to the processing and transport of the bacteriocin lactococcin-G which were detected in *Lb. plantarum* genomes. Moreover, multiple genes encoding for hydrogen peroxide were recognised in the genomes of both species. A large number of genes encoding for different virulence factors were observed in the pathogenic genomes. The majority of these genes were detected in all the three pathogens, except some other genes associated with the virulence of the individual pathogen. For example, staphylocoagulase in *S. aureus* (SA1) and streptolysin O in both *S. pyogenes* (SPA1) and *S. dysgalactiae* subsp *equisimilis* (SDG4).

Since the compared sequences of different bacterial species revealed that the 16S rRNA gene is highly conserved within a species and among some species of the same genus, it is

considered as a “gold standard” technique for determination of different species (Woo *et al.* 2002). Although phenotypic procedures can differentiate between two different bacterial species, 16S rRNA gene sequence analysis has more capacity to distinguish among species. Moreover, this analysis can assist for more accurate identification of species which are infrequently isolated, poorly defined, or even those with atypical characteristics (Clarridge 2004). Therefore, 16S rRNA genes have been increasingly applied in numerous investigations associated with bacterial taxonomy, evolution and phylogeny (Rajendhran and Gunasekaran 2011). PCR products obtained from the amplified 16S rRNA genes of *Lactobacillus* isolates and clinical pathogens involved in this study, all showed clear bands of approximately 1500 bp. Two important points should be taken into consideration when performing the homology searches for species identification in the databases. Firstly, the full length of 16S rRNA genes sequences is required to be at least 1500 bp. Secondly, the percentage coverage of the best hit has also a significant impact on the classification (Rajendhran and Gunasekaran 2011). Food lactobacilli isolated in the recent study were identified into two species *Lb. delbrueckii* and *Lb. plantarum*. Further investigation is required to differentiate between strains of the individual species. Various marker genes have been suggested to discriminate between species within the same genus. Nevertheless, only partial region of the genome is covered by a single locus sequence, thus, it is believed that the discriminatory capacity of this sequence has minor importance. To develop this power, sequencing of numerous conserved genes such as, multilocus sequence typing (MLST) in the bacterial genome has been recommended and is currently in practical applications for microbial phylogenetic investigation (Gevers *et al.* 2005; Rajendhran and Gunasekaran 2011). In this technique, the DNA sequences of internal fragments (nearly 500 bp length) of multiple housekeeping genes (usually seven) are used to distinguish between strains belonging to a single bacterial species and the phylogenetic analysis can be conducted by using the combined sequence profiles (Jolley *et al.* 2004). The group of housekeeping genes are selected depending on the sequence variability among the specific bacterial species (Rajendhran and Gunasekaran 2011). To expand the findings obtained in this chapter, additional research is essential to outline the industrial features of food isolates. For example, bacteriocin production and other probiotic capabilities which enable the selection of specific isolates as promising starter cultures to stimulate the fermentation process and deliver improved quality final products.

7.3 Investigation of the Bacterial Biodiversity of Fermented Food Products (Chapter 4)

The aim of this chapter was to explore the food bacterial community of fermented products, particularly LAB species. This was investigated by the NGS technique of the 16S rRNA gene sequences conducted for food samples including, yogurt and olives. Moreover, this involved examination of using different cultural media and incubation conditions to find out their effects on the bacterial diversity of food samples. In addition, exploration of the food LAB community by the NGS analysis, allowed to compare between the culture-independent method (NGS) and culture-dependent procedure performed for food samples in Chapter 3.

The findings of this research showed that extraction of genomic DNAs from both yogurt and Altunsa olive samples was successful. This was detected by the visualised obvious bands of PCR products in gel electrophoresis. Whereas the absence of a clear band for Zer olive PCR product was an indication of the difficulty encountered during the DNA extraction. The inability to extract the genomic DNA from this sample was due to hardness of the olive fruit flesh or may be the low levels of bacterial biomass present. Identification and classification of pure culture isolates is the first main application of 16S rRNA gene sequence analysis, which was carried out in Chapter 3. The second major application is assessment of the bacterial diversity in environmental samples without culturing by metagenomic methods (Rajendhran and Gunasekaran 2011). The 16S rRNA gene directly amplified and sequenced could deliver a more descriptive interpretation of a microbial community diversity than standard pure culture procedures (Rajendhran and Gunasekaran 2011).

The NGS analysis results revealed that Bacillaceae (which belongs to the Firmicutes phylum) was the main family found in all yogurt samples with a high prevalence of different *Bacillus* species irrespective of the medium and incubation condition used. With respect to *Lactobacillus* species, *Lb. delbrueckii* which was isolated from yogurt by cultivation in MRS medium (Chapter 3), this species was detected by NGS in low abundance as *Lb. delbrueckii* subsp. *indicus* within the sample of yogurt anaerobically incubated in MRS. Small percentages of other *Lactobacillus* species were also found in some yogurt samples. Furthermore, the NGS analysis showed that Lactobacillaceae family (which belongs to the Firmicutes phylum) was predominant in all olive samples regardless the medium and incubation condition differences. Several *Lactobacillus* species were observed in different ratios within these samples such as *Lb. rapi* which revealed the highest percentage of prevalence in FA medium incubated

anaerobically. Although *Lb. plantarum* was isolated from olives in Chapter 3 using MRS medium, it was not detected in any sample by the NGS technique. These findings suggested the variance between culture-dependent and culture-independent methods. It has been shown by Ceuppens *et al.* (2014) that differences between cultural and molecular procedures are usually detected in food microbiology. Clarridge (2004) declared that identification of non-cultured bacterial species can be conducted by the analysis of 16S rRNA gene sequences which enables the independence from cultivation conditions. Several factors allow this technology to compete and replace other conventional methods used for microbial identification. These factors involve, the existence of massive improved databases, developed DNA sequencing methods, and ready software and kits (Clarridge 2004). Tang and colleagues detected that fastidious bacterial isolates could be identified by 16S rRNA gene sequences, since this analysis allows more rapid and obvious recognition than other identification methods including, conventional biochemical procedures and carbon source utilization. Moreover, identification using 16S rRNA gene analysis can improve the results recovered from clinical investigations (Tang *et al.* 1998; Clarridge 2004).

The 16S rRNA gene sequences resulted from food samples were analysed using MiSeq standard operating procedure (SOP) pipeline of the Mothur bioinformatic software. Ceuppens *et al.* (2017) declared that involvement of mock and approved communities in the sequencing and analysis steps would be a useful training to evaluate strength and accuracy of a given pipeline. Involvement of synthetic communities in 16S rRNA gene amplicon sequencing has further advantage in the accurate assessment of samples, recognition of sequencing run biases and ideal filtering (Shakya *et al.* 2013; Poretsky *et al.* 2014). Primers used in the recent study targeted the regions V1 and V2 of the 16S rRNA gene, therefore, most of the obtained sequences in the recent study were identified at the species and only three sequences were classified to the genus level. Despite the short 16S rRNA gene fragments, Guo and colleagues reported that the maximum taxonomic accuracy of the species level is acquired for sequences covering the variable regions V1 and V2 of the 16S rRNA gene. However, less than half of the sequences are likely to be classified to the genus level (Guo *et al.* 2013; Ceuppens *et al.* 2017). The typical cultivation conditions of most bacterial species existed in a specific environmental niche are not fully recognised; therefore, the majority of these species are uncultivatable in the laboratory. As a result of that, direct extraction of the DNA from environment was analysed for 16S rRNA genes to investigate the biodiversity of microbes without culturing (Gill *et al.* 2006; Rajendhran and Gunasekaran 2008). Handelsman (2004) acknowledged that analysis of microbial community

by uncultured techniques revealed wide-ranging diversity than what was expected from cultured-based procedures. It was found that only around 1% of the total microbes could be cultivated on culture media in comparison to the actual microbial community obtained by metagenomic techniques (Rajendhran and Gunasekaran 2011). Furthermore, it was detected by Jenssen and co-workers that improvement of cultivation assays was infrequently achieved by understanding the phylogenetic diversity. Nonetheless, evaluation of the entire microbial community within any ecosystem is a continuing challenge (Janssen *et al.* 2002).

Overall, the results of this research demonstrated that similar percentages of bacterial species were detected in both yogurt and olive samples by the two databases used including, SILVA and RDP. In addition, the NGS analysis showed that the bacterial community as a whole was not influenced by the change in culture media or incubation conditions. Nevertheless, an increase in the abundance of *Lb. delbrueckii* subsp. *indicus* was observed in the yogurt sample incubated anaerobically in MRS medium, compared to the species prevalence under aerobic conditions.

In a previous study, a comparison between 16S rRNA gene-based analysis and metagenomic methodology has been conducted for synthetic communities (Shakya *et al.* 2013). Therefore, it might value to apply metagenomic approaches in the future to explore the microbial community of food samples included in the recent study and compare the findings of both classification methods. Despite the challenge to precisely conclude the taxonomic origin from metagenomic data (Bazinet and Cummings 2012), microbial communities in various schemes have been frequently described using metagenomic techniques (Rodriguez-Brito *et al.* 2010; Burke *et al.* 2011). A robust evaluation of the microbial community structure and diversity could be achieved by application of WGS metagenomic methods without the requirement of targeting and amplification of a single gene which may result in biased observations (Poretsky *et al.* 2014). Nevertheless, discrepancies in DNA preparation procedures, samples complication and the programmes used for sequencing can probably lead to differences in the phylogenetic classification of microorganisms in a specific community (Aird *et al.* 2011; Poretsky *et al.* 2014). In comparison to genomic databases, numerous inclusive and organised 16S databases are present in high quality. However, there is still a limitation in 16S rRNA gene sequencing technology which resulted from the inadequate databases used for sequence comparisons and the scarcity of 16S rRNA gene reads that could be definitely allocated to a genus level (Poretsky *et al.* 2014). Werner and co-workers declared that existence of sufficient reference genomes and gene sequencing could reduce the reliability on 16S rRNA gene amplicons for the determination

of genus-level. The low reliability is due to the biased power of the selected region of 16S rRNA gene which may result in discrepancies in assessed diversity levels (Claesson *et al.* 2010; Werner *et al.* 2012). Moreover, significant level of bacterial diversity can be masked since the 16S rRNA gene is highly conserved among bacterial species (Poretsky *et al.* 2014). More details of the community configuration could be identified by using metagenomic sequences for taxonomy compared to 16S rRNA gene amplicon sequences alone (Poretsky *et al.* 2014). Furthermore, 16S rRNA gene fragments retrieved in metagenomic reads are allowed to be studied with no necessity for the amplification step, in addition to the description of the full-length 16S rRNA gene sequences (Poretsky *et al.* 2014).

7.4 Determination of the Antibacterial Activity of Lactobacilli against Skin Pathogens *In Vitro* (Chapter 5)

The aim of this chapter was to examine the inhibitory effect of all *Lactobacillus* species including both food isolates and type strains against the growth of skin pathogens using the overlay method. Furthermore, this included investigations into the impact of *Lactobacillus* isolation source, the incubation time and conditions on the antagonistic activity. Further examination was performed to recognise the bioactive substances produced by lactobacilli which may play a role in preventing the pathogenic growth.

Findings reported in this chapter revealed that both food isolates and type strains had a significant inhibitory activity against all the three pathogens SA1, SPA1 and SDG4. The antibacterial potency was observed at the first day of incubation and increased gradually through incubation days, reaching the highest activity at day three of incubation. The increased inhibitory effect was detected under both aerobic and anaerobic incubation conditions. However, the inhibitory efficiency of most *Lactobacillus* species under anaerobic conditions was higher than the activity shown aerobically. Furthermore, the antibacterial effect for food-isolated lactobacilli was higher than that obtained by the type strains. Conclusions reported in this chapter depended on significant differences obtained by the statistical analysis. Overall, food-born *Lactobacillus* species showed higher effectiveness in preventing the growth of pathogens after three days of anaerobic incubation than type strains. Exploration of bioactive compounds which may be responsible for inhibiting growth, showed that the antagonistic activity was not only due to the organic acids production, but resulted from other produced antibacterial compounds potentially including bacteriocins. This non-acid based activity was detected by the comparison of the

inhibitory efficiency of *Lactobacillus* cultivated on buffered and non-buffered MRS media. The antibacterial activity of food-based lactobacilli obtained from *in vitro* experiments, allowed further investigations for their therapeutic activity against infections *in vivo,* which have been carried out in the next chapter (Chapter 6).

Further investigations are required to discover the antibacterial substances responsible for antagonistic effect shown by *Lactobacillus* species used in this research. Moreover, food *Lactobacillus* isolates or their recognised inhibitory compounds could be effectively applied as food preservatives to prevent the growth of food poisoning bacteria or other pathogens growing in the food material. New viewpoints for investigation would be opened by the application of new supplements to dairy and other food products, which can encourage the growth of lactic acid bacteria and enhance their inhibitory effect against a large number of pathogenic species. Further investigation is required to explore the synergistic antibacterial effect of a mixture of different lactic acid bacterial species against pathogenic bacteria involved in the present study.

7.5 Evaluation of the Therapeutic Potency of *Lactobacillus* Against Infections Caused by Skin Pathogens *In Vivo* (Chapter 6)

The aim of this chapter was to assess the survival percentages of the *in vivo* model, *Galleria mellonella,* in response to the injection of several treatments of *Lactobacillus* species isolated from fermented foods to determine the bacterial dose which revealed the highest survival of larvae. Different treatments of pathogens were also injected inside the larvae to distinguish the bacterial dose with the maximum lethal effect on the larvae. The main goal of this chapter was to explore the therapeutic potency of *Lactobacillus* against skin diseases by the common method of infection, the injection into the haemocoel. For further investigations, the healing effectiveness of food-isolated lactobacilli was explored using a novel infection technique by the topical application of pathogens on the larval dorsum followed by the application of *Lactobacillus.*

The results in this chapter showed that the high number of *Lactobacillus* cells injected in the larvae resulted in the lowest survival percentages. Despite of their probiotic characteristics, *Lactobacillus* revealed a virulent effect on the larvae when injected in high doses, regardless of whether the bacteria were injected as bacterial suspensions or washed cells. Furthermore, both bacterial suspensions and washed cells of *Lactobacillus* presented similar survival rates of larvae, while the lethal effect of the pathogenic suspensions was higher than that obtained from

the washed cells. As a consequence, the LD_{50} value of bacterial suspensions was higher than that of washed cells. An interesting result was observed when the LD_{50} value could be only determined by increasing the incubation time of larvae injected with *S. pyogenes* washed cells. Moreover, this value was not recognised for the injected *S. dysgalactiae* subsp. *equisimilis* washed cell even after extending the time of incubation, showing that the virulence of both pathogens might be decreased due to the reduction in the genetic material as a result of repeated cultivation procedures.

In terms of the therapeutic activity of food derived *Lactobacillus* species against infections caused by pathogens, the findings of this chapter showed that both *Lb. delbrueckii* and *Lb. plantarum* were effective in increasing the survival percentages of larvae previously infected with pathogens, indicating the therapeutic potential of these food isolates. A reduction in *S. aureus* and *S. pyogenes* infections was detected after the injection of both selected doses of *Lb. delbrueckii*, while the low injected dose of this species presented higher impact than the high dose in increasing the infection caused by *S. dysgalactiae* subsp. *equisimilis*. Regarding the effect of *Lb. plantarum* against the infections, the two doses chosen for the challenge experiment could decrease the infection of *S. aureus*, with more efficiency for the low dose of the food isolate. Nevertheless, both doses were not able to treat *S. pyogenes* infection, with a minor reduction of the lethality of low injected pathogenic doses. With respect to the novel trial of infection used, larvae topically infected with *S. pyogenes* followed by the topical treatment of washed cells and undiluted CFS of *Lb. plantarum*, both treatments presented an ability to decrease the death percentages of treated larvae in comparison to untreated groups. However, the effectiveness of washed cells was higher than that of the CFS.

In summary, the infection method and *Lactobacillus* mode of delivery had an impact on the progress of bacterial infection using the Greater wax moth larvae. Regarding the injection method, the two food isolates showed a significant therapeutic activity by increasing the survival percentages of infected larvae. Though, their effect against pathogenic infections depended on the infected pathogenic dose and the injected dose of *Lactobacillus*. Despite the therapeutic effect of *Lactobacillus* species included in the present study, it is not a simple task to choose and examine a microorganism for the effective application in humans and animals. The guidelines for probiotics evaluation in food have been framed by FAO/WHO practiced consultations in 2001 and 2002 (Food_and_Agriculture_Organization_of_the_United_Nations and World_Health_Organization 2001, 2002). Selection and estimation of probiotics as different formulations are recently supported by these guidelines, for instance, as a food supplement, a

food product or as a medicine used to prevent or treat specific infections (Reid *et al.* 2006; Hill *et al.* 2014). Al-Ghazzewi and Tester (2014) stated that food, drinks and capsules are the popular routes for probiotic application delivered to the gastrointestinal tract. Alternatively, topical application is progressively being studied (Al-Ghazzewi and Tester 2014). Köhler (2015) stated that the target organism has to be safe and efficient i.e., human clinical investigations are required for human probiotics. A preliminary stimulation for health applications can be only delivered by *in vitro* experiments of probiotic candidates or examinations in model organisms. Nonetheless, such investigations can provide an understanding of the potential mechanisms of probiotic activity (Food_and_Agriculture_Organization_of_the_United_Nations and World_Health_Organization 2001, 2002).

In this study, *Lactobacillus* species involved in the experiments *in vivo*, were isolated from fermented yogurt and olives. Thus, these food isolation sources allowed the safe injection of isolates inside the Greater wax moth larvae, and also enabled investigation of their therapeutic potential by the topical application on the larval dorsum. Moreover, positive results obtained from the antibacterial assay *in vitro* (Chapter 5) encouraged for the exploration of their potency against skin pathogens using the model *in vivo*. Although the antibacterial activity of *Lactobacillus* species has been shown in this study, it is important to perform other investigations *in vitro* to confirm that *Lactobacillus* isolates could be used or consumed as probiotics by humans. In addition to the antimicrobial effect, probiotic expert panels such as International Scientific Association for Probiotics and Prebiotics (ISAPP), recommended several *in vitro* studies, such as adherence capability to the mucosal surfaces of the intestine, resistance to gastric acids and tolerance to bile salts (Food_and_Agriculture_Organization_of_the_United_Nations and World_Health_Organization 2001, 2002). It has been stated in several studies, investigating probiotics, that a probiotic strain must have the ability to pass through the host digestive tract and remain alive to utilise its advantages on the host. The major focus of these studies was to investigate the sensitivity of strains to proteolytic enzymes, bile salts, and low pH (Charteris *et al.* 1998; Jacobsen *et al.* 1999). This research suggested the initial evidence of reducing the pathogenic infection by the effect of beneficial bacteria, however, several queries associated with the probiotic mechanisms in *Galleria mellonella* larvae are still to be uncovered. Köhler (2015) listed a number of questions about a previous experiments carried out by Vilela and co-workers who co-injected the larvae with the pathogenic yeast *Candida albicans* and a strain of *Lb. acidophilus* (Vilela et al. 2015). These questions including, how do lactobacilli reduce the pathogenic infection in the larvae? what is the function of the wax worm immune system? are

the results correlated to probiotic study in humans? Similar queries have been also brought up after performance of *in vivo* injection experiments in the present research. Using replicates per individual treatment is considered an important point, as it allows to better determine both advantages and limitations of the infection technique used. Each co-injection experimentation has been carried out in duplicate. To perform the injection experiment in duplicate, a total of 420 larvae were injected, and the whole injection procedure was continuously conducted for 9 - 12 hours. Therefore, performance of experiments using more replicates would be considered as logistically difficult and may result in experimental mistakes. Despite the interesting findings of injection technique, studying the development of bacterial infection and the interaction with delivered probiotics inside *Galleria mellonella*, is an area of research that needs to be expanded upon.

Since both gut and skin are considered as protective barriers from pathogenic infections, their environments have similar mechanisms of defence (Gallo and Hooper 2012), we hypothesized that the skin barrier protections against pathogenic bacteria might be improved by the topical application of *Lactobacillus* species included in this study. As previously discussed in Chapter 6, a few points should be kept in mind when evaluating the findings achieved from the topical application trials. A continuous application of a new prepared washed cells solution has to be applied once a day on the infected area during the experiment time instead of applying it at only one time directly after the infection to confirm the obtained conclusions and to achieve more robust results. Furthermore, the monitoring period of the injury progress should be increased to a minimum ten days rather than six days to ensure the full efficacy of the applied *Lactobacillus* solution against infection caused by the pathogens. Moreover, the injured area was macroscopically examined through the experimentation days, however, further investigations are required such as cultural and microscopic descriptions of the injury. For instance, a sample could be taken from the injured area on each individual day and cultured on appropriate selective media to assess the viability of both *Lactobacillus* and pathogenic bacterial cells and to count the bacterial colonies. Quantification of bacteria could be also carried out by a qPCR method during specific times during the experiment, for genes unique to the pathogen and probiotic.

Topical infection of the larvae was conducted by application of different doses of pathogenic cells starting from the low bacterial dose to the high one, nevertheless, results were obtained by using the concentrated dose of *Lactobacillus* species cells and the concentrated CFS

of that species. Despite the promising findings of this trial, it is required to explore the therapeutic effect of this *Lactobacillus* species in lower bacterial doses such as 10^2 CFU/larva or 10^3 CFU/larva. Since these doses showed an observable therapeutic effectiveness against infection using the injection technique, it was interesting to explore their healing potency when they are topically applied on the infected area. Furthermore, CFS was applied as a concentrated solution suggesting its high acidity which could exert an antibacterial effect, but may irritate the scratched dorsum of larvae. Hence, using the diluted solution of CFS could be more efficient in reducing the progress of injury without being irritative. Performance of such experiment will require long working hours, in addition to the large number of larvae used, especially when control groups and replicates are included. As the topical application experiment has been performed for only one pathogen (*S. pyogenes*) and one *Lactobacillus* species (*Lb. plantarum*), it is worthy to assess the healing efficiency of *Lb. plantarum* against other skin pathogens involved in this research. Sikorska and Smoragiewicz (2013) declared that skin wound infections caused by *S. aureus* have been prevented in mice by using *Lb. plantarum* cells and supernatant. In addition to the evaluation of the other food *Lactobacillus* species (*Lb. delbrueckii*) when it topically applied on the larvae infected with these pathogens. Re-establishment of the microbial balance and prevention of infections could be achieved by introducing the beneficial bacteria into gastrointestinal tract (Vanderhoof and Young 1998). Depending on the same concept, pathogens can be replaced from the skin by the topical application of advantageous bacterial species in a pharmaceutically adequate carrier.

In conclusion, fermented food products, in this case purchased from Iraqi supermarkets, are valuable sources for the isolation of lactic acid bacteria, which are the most common probiotics with multiple health advantages. Furthermore, a significant antagonistic efficiency of food-derived *Lactobacillus* species against skin pathogenic bacteria has been well approved *in vitro* in this study, indicating the presence of bioactive compounds produced by these species. The existence of inhibitory substances was confirmed by characterisation of food *Lactobacillus* sequenced genomes, which revealed several genes encoding for antibacterial compounds, in addition to other genes responsible for probiotic characteristics. The Greater wax moth larvae is considered as a valuable *in vivo* model for investigating pathogenic infections by injection. Moreover, a significant therapeutic effect was detected for food-based *Lactobacillus* species against infections caused by skin pathogens in *G. mellonella* larvae. In addition to the injection technique, valued knowledge was obtained by the topical application of a skin pathogen on the dorsum of larvae, which followed by the treatment with topically applied food isolated

Lactobacillus species. By using this novel procedure, a remarkable reduction in larval death percentages was detected as a result of *Lactobacillus* treatment, suggesting the promising therapeutic potency of food-derived LAB against skin infections. Further investigations are required to confirm the findings achieved by the topical application technique using *G. mellonella* larvae, which could pave the way for application of probiotic bacteria as therapeutic formulations to treat skin diseases in humans. Future work would expand the work reported here in other animal models to investigate whether the probiotics were able to reduce the symptoms of infection from these pathogens.

7.6 References

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Appendices

Appendix 1 (Chapter 3): BASH script of the software pipeline used for the whole genome sequences (WGS) analysis of bacterial raw sequence data using CLIMB linux virtual servers

This takes 2 paired fastq files and processes them all the way via Prokka

sh WGS_bat.sh **AGRNA2_12_S12 S12** # replaces **$1** with **AGRNA2_12_S12** and **$2** with **S12** - this will change according to which input files are used

fastqc $1_R1_001.fastq.gz $1_R2_001.fastq.gz

trim_galore --fastqc --paired **$1_R1_001**.fastq.gz **$1_R2_001**.fastq.gz

flash -d overlapped -o flash -z -M 150 -t 6 **$1_R1_001_val_1**.fq.gz **$1_R2_001_val_2**.fq.gz

spades.py -1 overlapped/flash.notCombined_1.fastq.gz -2 overlapped/flash.notCombined_2.fastq.gz -s overlapped/flash.extendedFrags.fastq.gz -k 33,55,99,111,125 --threads 6 -o duaa_**$2_spades**

bwa index duaa_**$2_spades**/contigs.fasta

bwa mem -v 3 -t 4 duaa_**$2_spades**/contigs.fasta **$1_R1_001_val_1**.fq.gz **$1_R2_001_val_2**.fq.gz | samtools sort --threads 2 -m 8G --reference duaa_**$2_spades**/contigs.fasta -o duaa_**$2_mapped**.bam

samtools index duaa_**$2_mapped**.bam

pilon --genome duaa_**$2_spades**/contigs.fasta --frags duaa_**$2_mapped**.bam --output duaa_**$2_corrected** --threads 6 --changes --mindepth 0.5

quast.py --gene-finding --threads 6 duaa_**$2_corrected**.fasta

prokka --force --outdir duaa_**$2_prokka** --force --prefix duaa_**$2** --compliant --centre CU --cpus 6 duaa_**$2_corrected**.fasta

Note: Programs used for the WGS analysis of bacterial raw sequence data are shown in red color.

Appendix 2 (Chapter 3): Genomes Assembly Stats

***Lactobacillus* Isolates**	**Genomes Assembly Stats**			
	Genome Size	**Genes Number**	**Contigs Number**	**CDS Number**
Lb15	2108757	2049	347	1961
Lb17	2116728	2042	369	1953
Lb18	2068553	2034	269	1946
Lb19	2033380	2025	207	1937
Lb20	3981587	3644	634	3558
Lb21	3607106	3338	230	3266
Lb22	3651137	3375	295	3301
Lb23	3590962	3328	221	3260
Lb24	3544508	3278	186	3210
Lb25	3975656	3709	264	3625

Lb: *Lactobacillus*. CDS: Coding Sequence

Appendix 3 (Chapter 4): Script of the MiSeq standard operating procedure (SOP) pipeline of the Mothur bioinformatics software package (v1.35.1) used for the next generation sequencing analysis of 16S rRNA gene amplicons from food samples using a Linux server

make.contigs(file=xx.files, processors=20)

summary.seqs(fasta=xx.trim.contigs.fasta, processors=20)

screen.seqs(fasta=xx.trim.contigs.fasta, group=xx.contigs.groups, summary=xx.trim.contigs.summary, maxambig=0, maxlength=xx, processors=20)

count.groups(group=xx.contigs.good.groups)

unique.seqs(fasta=xx.trim.contigs.good.fasta)

count.seqs(name=xx.trim.contigs.good.names, group=xx.contigs.good.groups)

align.seqs(fasta=xx.trim.contigs.good.unique.fasta, reference=/home/asmith8/silva.bacteria.fasta, flip=t, processors=20)

summary.seqs(fasta=xx.trim.contigs.good.unique.align,

count=xx.trim.contigs.good.count_table, processors=20) #optimize=end

screen.seqs(fasta=xx.trim.contigs.good.unique.align,

count=xx.trim.contigs.good.count_table,

summary=xx.trim.contigs.good.unique.summary, start=1044, end=6424, maxhomop=10, processors=20)

count.groups(count=xx.trim.contigs.good.good.count_table)

filter.seqs(fasta=xx.trim.contigs.good.unique.good.align, vertical=T, trump=.)

unique.seqs(fasta=xx.trim.contigs.good.unique.good.filter.fasta, count=xx.trim.contigs.good.good.count_table)

pre.cluster(fasta=xx.trim.contigs.good.unique.good.filter.unique.fasta, count=xx.trim.contigs.good.unique.good.filter.count_table, diffs=2, processors=20)

chimera.vsearch(fasta=xx.trim.contigs.good.unique.good.filter.unique.precluster.fasta, count=xx.trim.contigs.good.unique.good.filter.unique.precluster.count_table, dereplicate=t)

remove.seqs(fasta=xx.trim.contigs.good.unique.good.filter.unique.precluster.fasta, accnos=xx.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.accnos)

count.groups(count=xx.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.count_table)

classify.seqs(fasta=xx.trim.contigs.good.unique.good.filter.unique.precluster.pick.fasta,

count=xx.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.count_table, reference=../trainset16_022016.rdp.fasta, taxonomy=../trainset16_022016.rdp.tax, cutoff=80, method=wang, processors=20)

remove.lineage(fasta=xx.trim.contigs.good.unique.good.filter.unique.precluster.pick.fasta,
count=xx.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.count_table,

taxonomy=xx.trim.contigs.good.unique.good.filter.unique.precluster.pick.rdp.wang.taxonomy, taxon=Chloroplast-Mitochondria-unknown-Archaea-Eukaryota)

cluster.split(fasta=xx.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.fasta, count=xx.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.count_table, taxonomy=xx.trim.contigs.good.unique.good.filter.unique.precluster.pick.rdp.wang.pick.taxonomy, splitmethod=classify, taxlevel=4, cutoff=0.03, processors=20)

make.shared(list=xx.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.opti_mcc.unique_list.list, count=xx.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.count_table, label=0.03)

classify.otu(list=xx.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.opti_mcc.unique_list.list, count=xx.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.count_table, taxonomy=xx.trim.contigs.good.unique.good.filter.unique.precluster.pick.rdp.wang.pick.taxonomy, label=0.03)

count.groups(count=xx.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.count_table)

sub.sample(shared=xx.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.opti_mcc.unique_list.shared, size=xx)

#Rename the files

dist.seqs(fasta=xx.final.fasta, output=lt, processors=20)

clearcut(phylip=xx.final.phylip.dist)

collect.single(shared=xx.final.0.03.subsample.shared, calc=chao-invsimpson-shannon-npshannon, freq=1)

summary.single(calc=nseqs-sobs-chao-ace-invsimpson-npshannon-coverage-shannon)

unifrac.weighted(tree=xx.final.tre, name=xx.final.names, group=xx.final.groups, distance=square, processors=10, random=F, subsample=xx)

get.oturep(phylip=xx.final.phylip.dist, list=xx.final.list, fasta=xx.final.fasta, label=0.03)

#calculate taxonomy

java -Xmx1g -jar /home/ubuntu/mothur_monster/DB/RDPTools/classifier.jar classify -c 0.97 -o xx_classified.txt -h xx.txt --format=fixrank xx.final.0.03.rep.format.fasta

sudo usearch7.0.1090_i86linux64 -usearch_global xx.final.0.03.rep.format.fasta --db /home/ubuntu/DB/rdp_download_12227seqs.fa --uc xx_rdp97.txt --id 0.97 --maxaccepts 3 --maxrejects 0 --strand plus

Appendix 4 (Chapter 4): Next generation sequencing (NGS) of 16S rRNA gene amplicons from food samples showing bacterial diversity percentages of samples which were inoculated in two different media and incubated under two different conditions.

Phylum	Family	Species	1FA	1FAn	1MA	1MAn	3FA	3FAn	3MAn
Actinobacteria	Micromonosporaceae	*Actinoplanes globisporus* DSM43857	0	0	0	0.03	0.1	0.1	0.1
Actinobacteria	Bifidobacteriaceae	*Bifidobacterium animalis* subsp. *lactis* ATCC27673	0	0	0	0.1	0	0	0
Actinobacteria	Microbacteriaceae	*Leifsonia lichenia*	0	0	0	0	0	0.04	0.01
Firmicutes	Bacillaceae	*Bacillus cereus*	0	99.8	0.02	0.004	0.1	0.03	0
Firmicutes	Lactobacillaceae	*Lactobacillus rapi*	0	0	0.004	0.004	38	38.8	31.6
Firmicutes	Bacillaceae	*Bacillus licheniformis*	59.8	0.03	0.4	0.8	0.004	0.2	0
Firmicutes	Lactobacillaceae	*Lactobacillus buchneri* NRRL B-30929	0	0	0	0	28	20.7	11.7
Firmicutes	Bacillaceae	*Bacillus* sp. R-32842	0.04	0.02	99	82	0.009	0	0.004
Firmicutes	Lactobacillaceae	*Lactobacillus parafarraginis*	0.004	0	0	0	13.7	13	22.7
Firmicutes	Lactobacillaceae	*Lactobacillus pentosus*	0	0	0	0	6	5.7	24.8
Firmicutes	Bacillaceae	*Bacillus amyloliquefaciens*	35	0.009	0	0	0.03	0.01	0
Firmicutes	Lactobacillaceae	*Lactobacillus acidipiscis*	0	0	0	0.004	9.7	17.7	6.5
Firmicutes	Streptococcaceae	*Streptococcus thermophilus* LMD-9	0.04	0.03	0.08	13	0	0	0
Firmicutes	Paenibacillaceae	*Paenibacillus timonensis*	2	0	0	0	0.004	0	0
Firmicutes	Lactobacillaceae	*Pediococcus ethanolidurans*	0	0	0	0	0.6	0.5	1.2
Firmicutes	Paenibacillaceae	*Paenibacillus cookii*	1	0	0.01	0.1	0	0	0
Firmicutes	Lactobacillaceae	*Lactobacillus delbrueckii* subsp. *indicus*	0	0	0.01	0.8	0	0	0
Firmicutes	Paenibacillaceae	*Paenibacillus relictisesami*	0.7	0	0.02	0.03	0	0	0
Firmicutes	Lactobacillaceae	*Lactobacillus vaccinostercus*	0	0	0	0	0.2	0.3	0.1
Firmicutes	Bacillaceae	*Bacillus coagulans*	0	0	0.1	0.3	0	0	0
Firmicutes	Staphylococcaceae	*Staphylococcus aureus*	0	0	0	0.02	0.1	0.03	0.1
Firmicutes	Lactobacillaceae	*Lactobacillus* sp. ATCC_8291	0	0	0	0	0.1	0.1	0.01
Firmicutes	Lactobacillaceae	*Lactobacillus namurensis*	0	0	0	0	0.004	0.1	0.03
Firmicutes	Lactobacillaceae	*Pediococcus parvulus*	0	0	0	0	0.01	0.03	0.01
Proteobacteria	Pseudomonadaceae	*Pseudomonas aeruginosa*	0	0	0.02	1.1	1.8	2	0.9
Proteobacteria	Oxalobacteraceae	*Massilia timonae*	0	0	0	0.1	0.4	0.3	0.1
Proteobacteria	Burkholderiaceae	*Ralstonia solanacearum*	0	0	0	0.1	0.06	0.03	0

Proteobacteria	Comamonadaceae	*Delftia* sp. R-41380	0	0	0	0.1	0.04	0.03	0.004
Proteobacteria	Xanthomonadaceae	*Stenotrophomonas maltophilia*	0	0	0	0.1	0.07	0.03	0.04
Proteobacteria	Brucellaceae	*Ochrobactrum anthropi*	0	0	0	0	0.04	0.01	0
Proteobacteria	Moraxellaceae	*Acinetobacter pittii*	0	0	0	0	0.03	0.01	0.01
Proteobacteria	Enterobacteriaceae	*Shigella dysenteriae*	0.004	0	0	0.02	0.01	0.01	0.004
Unclassified	Unclassified	Unclassified	0	0	0	0.01	0.04	0.03	0.02

F: Facultative anaerobic, M: MRS, A: Aerobic, An: Anaerobic. Green cells: The most important data of yogurt samples. Yellow cells: The most important data of olive samples NGS analysis has been conducted by Mothur bioinformatics tool. Two databases: SILVA and RDP have been used for taxonomic binning and both showed similar taxonomic identification

Appendix 5 (Chapter 4): Script of the Phyloseq package used for the graphical display of operational taxonomic units (OTUs) of the next generation sequencing (NGS) of 16S rRNA gene amplicons from food samples using RStudio software (v1.2.1335)

```
# import data sets from excel


otu_table and tax_table


rownames(otumatx) <-paste0("OTU", 1:nrow(otumatx))


    colnames(otumatx) <-paste("Sample", 1:ncol(otumatx)) ## leave out if you want to keep the original sample numbers otherwise it changes the numbers to consecutive Sample1, Sample2 etc


otumatx


library(phyloseq)


OTU = otu_table(otumatx, taxa_are_rows = TRUE)


OUT


rownames(TAX) <- rownames(OTU)


TAX
```

```
colnames(TAX) <-c("Phylum", "Class", "Order", "Family", "Genus", "Species")

TAX

physeq=phyloseq(OTU,TAX)

physeq

plot_bar(physeq, fill = "Phylum")

plot_heatmap(physeq)
```

Appendix 6 (Chapter 5): Zones of growth inhibition (ZOI) diameters (mm) of *S. aureus* produced by lactobacilli using overlay method under two different incubation conditions (aerobically and anaerobically) after 24 h, 48 h and 72 h

Lactobacilli	Incubation's Conditions	Zones of Growth Inhibition (mm)		
		24 h	48 h	72 h
Lb15	A	23	35	40
	An	32	48	53
Lb17	A	26	35	36
	An	37	47	54
Lb18	A	21	28	33
	An	30	42	47
Lb19	A	21	30	34
	An	31	40	45
Lb20	A	13	30	35
	An	16	41	46
Lb21	A	24	33	44
	An	29	50	55
Lb22	A	20	32	32
	An	25	46	50
Lb23	A	22	33	35
	An	25	47	47
Lb26	A	18	27	27
	An	28	40	42
Lb27	A	20	27	37
	An	27	30	48
Lb28	A	20	26	31
	An	30	36	46
Lb29	A	26	30	40
	An	35	45	50
Lb30	A	32	45	50
	An	40	47	50

Lb: *Lactobacillus*, A: Aerobic, An: Anaerobic

Appendix 7 (Chapter 5): Zones of growth inhibition (ZOI) diameters (mm) of *S. pyogenes* produced by lactobacilli using overlay method under two different incubation conditions (aerobically and anaerobically) after 24 h, 48 h and 72 h

Lactobacilli	Incubation's Conditions	Zones of Growth Inhibition (mm)		
		24 h	48 h	72 h
Lb15	A	22	31	46
	An	30	40	43
Lb17	A	25	32	40
	An	35	45	50
Lb18	A	21	31	35
	An	30	42	48
Lb19	A	20	28	33
	An	28	41	45
Lb20	A	13	30	32
	An	13	40	42
Lb21	A	26	32	40
	An	29	43	46
Lb22	A	22	32	35
	An	23	45	45
Lb23	A	24	31	32
	An	27	43	46
Lb26	A	17	23	25
	An	30	37	45
Lb27	A	20	35	35
	An	30	45	50
Lb28	A	20	25	35
	An	30	38	48
Lb29	A	24	33	40
	An	40	45	50
Lb30	A	30	50	50
	An	30	52	54

Lb: *Lactobacillus*, A: Aerobic, An: Anaerobic

Appendix 8 (Chapter 5): Zones of growth inhibition (ZOI) diameters (mm) of *S. dysgalactiae* subsp *equisimilis* produced by lactobacilli using overlay method under two different incubation conditions (aerobically and anaerobically) after 24 h, 48 h and 72 h

Lactobacilli	Incubation Conditions	Zones of Growth Inhibition (mm)		
		24 h	48 h	72 h
Lb15	A	30	32	40
	An	30	45	50
Lb17	A	35	33	40
	An	35	45	47
Lb18	A	28	30	35
	An	28	43	45
Lb19	A	28	28	35
	An	28	40	44
Lb20	A	17	30	35
	An	25	40	53
Lb21	A	27	40	45
	An	30	35	40
Lb22	A	27	45	40
	An	30	40	48
Lb23	A	25	30	40
	An	28	35	50
Lb26	A	26	35	44
	An	24	34	40
Lb27	A	30	38	48
	An	30	38	45
Lb28	A	28	30	36
	An	27	40	44
Lb29	A	30	40	42
	An	35	45	50
Lb30	A	40	45	50
	An	40	45	50

Lb: *Lactobacillus*, A: Aerobic, An: Anaerobic

Appendix 9 (Chapter 5): Zones of growth inhibition (ZOI) diameters (mm) of pathogens produced by lactobacilli using overlay method after 72 h under aerobic incubation condition

Lactobacilli	Mean ± SD of Growth Inhibition's Zones (mm)		
	SA1	SPA1	SDG4
Lb1	25.7±1.2	22.7±0.6	27±2.7
Lb2	38.7±2.3	35.3±1.5	37.7±3.8
Lb6	43.7±1.5	38.7±1.2	38±2.0
Lb15	42.3±2.5	41±4.4	40±0
Lb17	38.7±2.3	39±1.7	40.7±1.2
Lb18	37.7±4.0	40.3±4.6	39.3±3.8
Lb19	40±5.3	36±3.0	40±5.0
Lb20	37±1.7	32.3±2.5	35±0
Lb21	41.3±2.3	36.7±2.9	41.7±3.5
Lb22	35.7±4.0	31.7±2.9	38±2.0
Lb23	35.7±1.2	36.7±4.2	38.3±1.5
Lb24	42.3±4.0	34.7±0.6	40.7±4.0
Lb25	45.3±4.2	40.3±0.6	38.7±1.2
Lb26	32.3±6.1	26.3±1.5	33±9.9
Lb27	36.7±1.5	33.3±1.5	45±3.0
Lb28	35.7±4.5	40±5.0	37.3±2.3
Lb29	48.3±7.6	41.7±2.9	40.3±2.1
Lb30	40±8.67	41.7±9.1	46±3.5
Lb32	12.3±1.5	11±0	4.7±8.1
Lb33	20±5.0	18.3±5.8	19.7±0.6

Lb: *Lactobacillus*

Appendix 10 (Chapter 5): Zones of growth inhibition (ZOI) diameters (mm) of pathogens produced by lactobacilli using overlay method after 72 h under anaerobic incubation conditions

Lactobacilli	Mean ± SD of Inhibition's Zones (mm)		
	SA1	SPA1	SDG4
Lb1	42.7±2.5	35±1.7	35.3±0.6
Lb2	49.3±1.2	41.3±1.2	51±1.7
Lb6	49.3±1.2	40±0	48.3±1.5
Lb15	49.3±3.2	41.7±1.5	49.7±0.6
Lb17	49±4.6	45.7±4.0	48.3±1.5
Lb18	46.3±1.2	46±1.7	51±5.3
Lb19	49±3.6	45±0	51.7±6.7
Lb20	37±8.2	34.3±6.7	45±7.0
Lb21	52.7±2.5	38.7±6.4	48.7±7.5
Lb22	41.7±7.2	36.7±7.6	39±7.9
Lb23	40±6.1	45±1.0	40.3±8.4
Lb24	48±1.7	38±1.7	49±1.7
Lb25	51.3±2.3	44.3±0.6	51.7±1.5
Lb26	36.3±5.1	33.7±9.8	33.3±5.8
Lb27	40±7.2	39.7±8.9	42.3±2.5
Lb28	40.7±4.6	45±3.0	42.7±2.5
Lb29	49.3±1.2	44.3±1.2	46±3.6
Lb30	46±3.6	45.7±8.0	47.3±3.1
Lb32	21.7±2.9	16.7±2.9	20.3±8.4
Lb33	34±1.0	32.3±2.5	31.7±2.9

Lb: *Lactobacillus*

Appendix 11 (Chapter 6): Script of the non-parametric analysis of variance (one-way ANOVA) for multiple groups **(**Dunn test Kruskal-Wallis test**)** used for the statistical analysis of the larval survival percentages using R Studio software (v3.6.1)

```
input the file


#select your tab delimited text file


my_data <- read.delim(file.choose())


head(my_data)


# you will need to install the following libraries


# they are all found from the Tools > Install Packages menu


library("FSA")


dunnTest(surv ~ treat, data = my_data, method="bh")
```