Molecular genetic characterisation of probiotic bacteria: *Lactobacillus acidophilus* and *Bifidobacterium* species

Thesis presented for the Degree of Philosophiae Doctor
By

Matthew Bull, BSc (Hons) Microbiology

In candidature for the Degree of Philosophiae Doctor

Organisms and the Environment Division
Cardiff School of Biosciences
Cardiff University

September 2013

DECLARATION

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed …… (candidate) Date: 30th September 2013

STATEMENT 1

This thesis is being submitted in partial fulfilment of the requirements for the degree of PhD.

Signed …… (candidate) Date: 30th September 2013

STATEMENT 2

This thesis is the result of my own independent work/investigation, except where otherwise stated.

Other sources are acknowledged by explicit references.

Signed …… (candidate) Date: 30th September 2013

STATEMENT 3

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed …… (candidate) Date: 30th September 2013

STATEMENT 4

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loans **after expiry of a bar on access previously approved by the Graduate Development Committee.**

Signed …… (candidate) Date: 30th September 2013

ACKNOWLEDGEMENTS

I would like to sincerely thank the following people for their help and support throughout my PhD studies:

- Prof. Esh Mahenthiralingam and Dr. Julian Marchesi for their excellent supervision, guidance and patience at all times.

- Dr. Sue Plummer and Dr. Iveta Garaiova, from Cultech Ltd. for their expertise and enthusiasm for the project.

- Prof Martin Maiden, Dr. Keith Jolley and Dr. Holly Bratcher for their assistance in resequencing, assembling and comparing *Lactobacillus* genomes.

- Prof. Peter Vandamme and Dr. Maarten Aerts, for the provision of LAB isolates and training on MALDI-TOF mass spectrometry.

- The BBSRC for funding this studentship (Doctoral Training Grant BB/F016557/1), and Cultech Ltd. for additional funding as CASE sponsors of this project.

- My mum Anne, and brother Tim, for their help and support.

- My friends and colleagues at Cardiff University; Dr. Laura Rushton, James Evans, Laura Morris, Beky Weiser, Dr. Phil Norville and Dr. Judith White.

SCIENTIFIC CONFERENCES AND AWARDS

Analysis of the strain diversity of *Lactobacillus acidophilus*: an important probiotic bacterium (2010) South Wales and South West Microbiology Forum. Cardiff University, Wales, UK. Oral and poster presentation

Molecular identification and genetic diversity of the probiotic species *Lactobacillus acidophilus* (2011) FEMS congress of European Microbiologists. Geneva, Switzerland. Poster presentation.

Strain-level diversity analysis of *Lactobacillus acidophilus* reveals a domesticated, clonal probiotic species with limited host range (2012) Society for General Microbiology Spring Meeting. Dublin, Ireland. Poster presentation. Awarded a Society for General Microbiology travel grant

Strain-level diversity analysis of *Lactobacillus acidophilus* reveals evidence of a clonal probiotic species with high carriage rate in humans (2012) All Wales and South West Microbiology Forum. Oral presentation. Awarded the Society for General Microbiology Communication Prize

Clonality of the bacterial probiotic species *Lactobacillus acidophilus* (2012) Microbiology and Infection Translational Research Group (MITReG) Postgraduate Day. Swansea University, Wales, UK. Oral presentation. Awarded 1st prize for oral presentations.

Strain-level diversity analysis of *Lactobacillus acidophilus* reveals evidence of a clonal probiotic species with high carriage rate in humans (2013) FEMS congress of European Microbiologists. Leipzig, Germany. Poster presentation. Awarded a Society for General Microbiology travel grant

PUBLICATIONS

Bull, M. J., Marchesi, J. R., Vandamme, P., Plummer, S. & Mahenthiralingam, E. (2012) Minimum taxonomic criteria for bacterial genome sequence depositions and announcements. Journal of Microbiological Methods, 89 (1) 18-21.

Baxter, C. G., Rautemaa, R., Jones, A. M., Webb, A. K., Bull, M., Mahenthiralingam, E. & Denning, D. W. (2013) Intravenous antibiotics reduce the presence of *Aspergillus* in adult cystic fibrosis sputum. Thorax, 68 (7) 652-657.

Bull, M. J., Plummer, S., Marchesi, J. and Mahenthiralingam, E. (2013) The life history of a commercial probiotic: The revisionary taxonomy and physiological characteristics of *Lactobacillus acidophilus*. (Submitted to FEMS Microbiology Letters).

SUMMARY

Introduction

Over time and concurrent development of methods to identify and characterise bacteria, the lactic acid bacteria (LAB) have undergone multiple taxonomic revisions. As a result of the revisionary nature of LAB taxonomy, the historical characterisation of *Lactobacillus acidophilus* has struggled with misidentification and misrepresentation. Now however, due to its global use in food products for both flavour and probiotic effect, *L. acidophilus* is now one of the most well physiologically characterised *Lactobacillus* species. *Bifidobacterium bifidum* and *Bifidobacterium animalis* subsp. *lactis* are also LAB that are considered to have probiotic effects. Here modern high-throughput next generation comparative genomic techniques are used alongside conventional biochemical and molecular typing methods to analyse the sub-species level diversity of these three probiotic species.

Results

Randomly Amplified Polymorphic DNA (RAPD) profile similarity analysis showed limited strain-level diversity of *L. acidophilus*. A species specific marker test was developed for *L. acidophilus* and used to search for *L. acidophilus* in wild rodent and human faeces. No *L. acidophilus* was detected in wild rodent faeces and its carriage in human faeces was highly variable. High-throughput next generation sequencing was used to resequence the genomes of 28 *L. acidophilus* isolates. Comparing these sequences indicated a high level of genomic conservation in *L. acidophilus*, which was reflected by limited phenotypic diversity. Comparative genomics in *Bifidobacterium animalis* subsp. *lactis* supported the hypothesis that it is a clonally monophyletic species, whereas *B. bifidum* strains were genomically diverse.

Conclusions

Methods for phenotypically characterising and typing LAB have generally been superseded in accuracy by DNA sequence based methods. Probiotic bacteria display a range of subspecies level population structures. Commercial and culture collection *L. acidophilus* isolates did not significantly differ phenotypically, but were distinct when their genome sequences are compared. *B. bifidum* was genotypically diverse at the subspecies level, while *B. animalis* subsp. *lactis* appeared to be clonally monophyletic. Comparative genomics and genome (re)sequencing of probiotic bacteria will become a “gold standard” method for characterisation and typing of isolates.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABC	ATP binding cassette
CAN	Acetonitrile
ATCC	American Type Culture Collection
BIGSdb	Bacterial Isolate Genome Sequence Database
BLAST	Basic local alignment tool
bp	Base pairs
CDS	Coding sequences
cfu	colony forming units
CHCA	α-cyano-4-hydroxycinnamic acid
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
DSM	(DSMZ) German collection of Microoragnisms and Cell Cultures
EDTA	Ethylenediaminetetraacetic acid
EPS	Exopolysaccharide
EU	European Union
F6PPK	fructose-6-phosphoketolase
FAO	Food and Agriculture Organisation
FDA	Foods and Drugs Administration
FIC	Fractional inhibitory concentration
FOS	Fructooligosaccharide
GC	Guanine-Cytosine

GIT	Gastrointestinal Tract
GOS	Galactooligosaccharide
GRAS	Generally Regarded As Safe
HGT	Horizontal gene transfer
HMO	Human Milk Oligosaccharide
IBD	Inflammatory Bowel Disease
IMG	Integrated Microbial Genomes
LAB	Lactic Acid Bacteria
Lab4®	Cultech Ltd. probiotic supplement
LMG	Belgian co-ordinated collections of micro-organisms, Gent.
Nd:YAG	Neodymium-doped yttrium aluminum garnet
NGS	Next Generation Sequencing
m/z	Mass-to-charge (ratio)
MALDI-TOF	Matrix Assisted Laser Desorption/Ionisation - Time Of Flight
MLST	Multi-locus sequence typing
MMS	Methylmethane Sulphonate
MRS	de Man, Rogosa and Sharpe (agar / broth)
MS	Mass spectrometry
NADH	Nicotinamide adenine dinucleotide
NCIMB	National Collection of Industrial, Food and Marine Bacteria, UK
NCBI	National Centre of Biotechnology and Information
NCTC	National Collection of Type Cultures
NJ	Neighbour Joining
nMDS	Non-metic multi-dimensional scaling
ORF	Open Reading Frame
PAU	Potential Autonomous Unit

PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
PROSAFE	Biosafety Assessment of Probiotics used for Human Consumption
R	R statistical software
RAPD	Random amplified polymorphic DNA
RDP	Ribosomal Database Project
rep	repetitive extragenic palindromic
RFLP	Restricted fragment length polymorphism
rMLST	Ribosomal Multi Locus Sequence Typing
RNA	ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
SNPs	Single Nucleotide Polymorphisms
Taq	*Thermus aquaticus*
TFA	trifluoroacetic acid
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UTI	Urinary Tract Infection
V	Volt
WHO	World Health Organisation

1. INTRODUCTION

1.1. PROBIOTIC BACTERIA – A GENERAL INTRODUCTION

The indigenous microbiota of the human gut is a heterogeneous community, considered to harbour more than 500 bacterial species (Blaut et al., 2002). One of the primary functions of this population, at least from the point of view of the host, is to prevent the colonisation of the host gut by pathogenic microorganisms. This is achieved in the most part by out-competing any potential invasive pathogens for metabolic substrates that are abundant in the colon (Tuohy et al., 2003). This system for prevention of gut colonisation by pathogens is not infallible however, and may be overcome by infection with specific gut pathogens, such as *Salmonella* species. The gut also may be at risk of colonisation by potential pathogens in compromised individuals, such as those suffering from bowel cancer or Inflammatory Bowel Disease (IBD), and individuals that have gut microbiota compromised by antibiotics. The recognition of these factors led to the development of foods and supplements that are specifically designed to reinforce the host gut microbiota in the face of perturbations by pathogens. These foods and supplements often contain microorganisms termed probiotics.

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a beneficial effect on the health of the host (FAO/WHO, 2001). Furthermore, this definition is expanded upon to state that probiotic organisms used in food must be capable of surviving passage through the gut. They must therefore be tolerant to gastric juices and exposure to bile. In addition, they must be safe and effective, and maintain their effectiveness and potency for the duration of the shelf-life of the product (FAO/WHO, 2002).

1.1.1. DELIVERY OF PROBIOTICS

The variety in manufacturing processes enables the delivery of probiotics to the consumer in numerous ways, including dairy foods such as fermented milks and cheeses and non-dairy foods like cereals, to freeze-dried powders, with each delivery matrix contributing

differently to the use and probiotic effect of the product. Delivery matrices may influence probiotic functionality in numerous ways; including induction of changes in the cell composition and physiology of the probiotic, provision of bioactive compounds, delivery of fermentation end products such as organic acids and secondary metabolites like bateriocins. The palatability of the delivery matrix can also alter the frequency at which probiotic products are consumed and incorporated into the diet (Sanders and Marco, 2010). Reasonably, all of the above factors could affect product shelf-life and stability, and probiotic cell fitness, directly impacting on the quantity of active probiotic delivered to the consumer, which forms a vital part of the manufacturers label claim (Sanders, 2008).

1.1.2. HISTORY OF PROBIOTICS AND PROBIOTIC FOOD PRODUCTS

The earliest observation of the positive role played by some bacteria was made in 1907 (Metchnikoff, 1907). Metchnikoff hypothesized that the long and healthy lives of Bulgarian peasants were due to their consumption of large quantities of fermented milk products containing bacteria. The term “probiotic” was initially proposed as an alternative to the term “antibiotic”, to describe substances secreted from microorganisms that promoted the growth of another rather than retarded it (Lilly and Stillwell, 1965). The term probiotic was subsequently redefined to “organisms and substances which contribute to intestinal microbial balance”, more closely analogous its meaning today (Parker, 1974). Changes to the definition were made again in 1989 (Fuller), 1992 (Havenaar and Huis in’t Veld) and 1996 (Schaafsma). These definitions were generally made and refined by individuals, rather than the most recent FAO/WHO definition, which was agreed upon at committee (FAO/WHO, 2002).

1.1.3. COMMON PROBIOTICS AND THEIR SELECTION CRITERIA

The choice of organisms to administer as probiotic supplements is largely historical and does not necessarily take into account the mode of therapeutic or prophylactic action of the organism. The historical definition of an organism as probiotic is largely based on years of administration to humans with no harmful side effects. Kopp-Hoolihan (2001) sets out a range of criteria that should be met by probiotic organisms:

1. The ability to exert a beneficial effect on the host
2. The ability to survive transit through the gastrointestinal tract
3. The ability to adhere to the intestinal epithelial cell lining
4. The ability to produce antimicrobial substances towards pathogens
5. The ability to stabilise the intestinal microbiota

Probiotics added to food generally meet at least one of these criteria. There is also a practical concern for companies that market probiotics, formulations must have a reasonable shelf-life and maintain a consistent number of viable organisms during on-shelf storage i.e. in between quality control checks and consumption. Table 1.1 lists organisms that are commonly added to commercial probiotic formulations intended for human consumption, and describes studies of their efficacy *in vivo*. The most widely used probiotic organisms belong to the Lactic Acid Bacteria (LAB) (section 1.2). The genera *Lactobacillus* and *Bifidobacterium* are the most extensively studied of the LAB.

Table 1.1 Bacterial species commonly used in commercial probiotic formulations.

Genus	Species	Comments	Reference
Lactobacillus	*acidophilus*	Strain LA-5 reduced antibiotic Associated diarrhoea	(Ouwehand et al., 2002)
	casei	Strain Shirota shortened rotavirus diarrhoea	
	fermentum		
	johnsonii	Strain LA-1 reduced colonisation by *Helicobacter pylori*	
	paracasei		
	plantarum	Strain 299v relieved symptoms of irritable bowel syndrome	
	rhamnosus	Strain GG shortened rotavirus diarrhoea	
	salivarius	Strain UCC118 relieved symptoms of irritable bowel syndrome	
Bifidobacterium	*breve*	Strain 299v relieved symptoms of irritable bowel syndrome	(Brigidi et al., 2001)
	longum		
	bifidum	Strain Bb12 shortened rotavirus diarrhoea	(Saavedra et al., 1994)
Enterococcus	*faecium*		
Saccharomyces	*boulardii*	Maintenance treatment of Crohn's disease	(Guslandi et al., 2000)

1.1.4. MODE OF ACTION AND CLINICAL IMPLICATIONS OF PROBIOTICS

There are many products available that contain probiotic bacteria, although little *in vivo* evidence exists to document the means by which probiotics confer a beneficial effect on the host. Therapeutically, probiotics have been used to modulate immunity, lower cholesterol, treat rheumatoid arthritis, prevent cancer, improve lactose intolerance, and prevent or reduce the effects of atopic dermatitis, Crohn's disease, diarrhoea, and constipation as well as candidiasis and urinary tract infections (UTI) (Reid, 1999).

The adherence of probiotic organisms to intestinal muscosal cells is observed *in vitro* (Greene and Klaenhammer, 1994) and again, is considered to be a vital property of probiotic organisms. Little however, is known about persistence times and processes associated with probiotics *in vitro*, and this is set to remain the case until a gut model that is suitably analogous to that of a human is developed. Adherence to intestinal cells may not occur *in vivo* at efficiencies suggested by *in vitro* experimentation, as probiotic feeding studies have shown short persistence times of *Lactobacillus acidophilus* administered within a probiotic capsule (Mahenthiralingam et al., 2009), and their application is certainly not followed by a high level of intestinal colonisation. Other health promoting aspects of probiotics include: the suggested sequestration of low-density lipoproteins (Taylor and Williams, 1998) implicated in increased risk of heart disease, improved lactose digestion in lactose intolerant individuals (Sanders, 1993) and immune enhancement in infants (Schiffrin et al., 1995).

The production of antimicrobial compounds is thought to be an important factor that allows specifically *Lactobacillus* spp. to competitively exclude harmful or pathogenic organisms from the human gut. The antimicrobial compounds produced by *Lactobacillus* species in oxygen depleted environments, such as the human gut, include lactic acid, diacetyl and β-hydroxypropionaldehyde. Also produced *in vitro* are bacteriocidal and bacteriostatic peptides that have the ability to influence the growth of numerous organisms, including members of the genus *Lactobacillus* (Sanders and Klaenhammer, 2001) and other, less closely related foodbourne, disease-causing organisms (Gilliland and Speck,

1977). The *in vivo* production of bacteriocins and bacteriostatic compounds and their subsequent role in the gut is much more poorly documented.

1.2. THE LACTIC ACID BACTERIA

LAB constitutes a diverse group of Gram positive, non-sporulating, catalase-negative organisms that are found in a number of habitats (Carr et al., 2002). LAB are comprised of multiple genera within the order *Lactobacilliales* that are acid tolerant, of which *Enterococcus*, *Streptococcus* and *Lactobacillus* species are among the most well characterised. They are known constituents of the human gut (Arumugam et al., 2011) and also occur widely in dairy, meat, plants and fermented products of commercial value (Carr et al., 2002). As a result of their ability to rapidly ferment carbohydrates to lactic acid, they have become industrially important bacteria and are used in a myriad of food and agricultural fermentations worldwide. Their growth causes acidification of food material, preserving the product and imparting unique textures and flavours (Kleerebezem and Hugenholtz, 2003). Healthy humans and animals are known to carry a number of LAB species within their lower digestive tract, and several LAB are now used as probiotics (Shah, 2007). The LAB are mostly associated with environments that are rich in nutrients, such as the human gut or various food products.

1.2.1. USES OF LAB

LAB have been used to create fermented food products for thousands of years, in particular they are used in the production of fermented milk products including kefir, yoghurt, cheeses and butter. The addition of LAB to create fermented dairy products centres on their ability to reduce the pH of the surrounding food matrix, combined with the production of bacteriocins and ethanol which inhibit the growth of other spoilage organisms and extends the shelf life of the product (Leroy and De Vuyst, 2004). Lactic acid production in milk-based products involves the fermentation of lactose to lactic acid. The presence of lactic acid in milk products causes alterations in the structure of the proteins present, in effect curdling the product. Other products of heterofermentative conversion of lactose to lactic acid, such as acetaldehyde and diacetyl, impart unique flavours to the products that contain them (Sanders et al., 1996). LAB also predominate in other non-dairy fermented food products such as sauerkraut, fermented sausages, fermented fish, pickles, sourdough and rice wine (Leroy and De Vuyst, 2004).

1.2.2. HISTORY AND TAXONOMY OF THE LACTOBACILLI

Lactobacillus is a highly heterogeneous genus, encompassing bacteria with a wide range of biochemical and physiological properties (Felis and Dellaglio, 2007, Salvetti et al., 2012). The genus *Lactobacillus* is the largest of those that belong to the LAB, with 185 species validly described at the time of writing, and increasing substantially from 145 in 2008 as a result of the reclassification of multiple species (Claesson et al., 2008, Euzéby, 1997).

From the initial description of the species *Lactobacillus acidophilus* in 1920 (Holland) until around 1970, many *Lactobacillus* isolates from human mucosal surfaces were collectively identified as *L. acidophilus* (Figure 1.1). The identification of isolates using traditional phenotypic characteristics such as the fermentation of carbohydrates and cellular morphology, combined with the lack of a robust taxonomical framework, had historically led to such *Lactobacillus* isolates being incorrectly designated at the genus level. The development of polyphasic taxonomic approaches and use of molecular/genomic systematics has greatly improved the classification of *Lactobacillus* species, and are a prerequisite characterisation step for probiotic lactobacilli marketed commercially (Vankerckhoven et al., 2008).

Traditionally, the identification of lactobacilli has been mostly undertaken phenotypically, based in the most part on the fermentation of carbohydrates, cellular morphology and Gram staining (Tynkkynen et al., 1999). Key phenotypic characteristics of lactobacilli include cells shaped as rods or coccobacilli, metabolism of carbohydrates by fermentation and microaerophilic oxygen requirements. They are chemoorganotrophic, requiring rich media for growth (Felis and Dellaglio, 2007). Phenotypic methods of identification are still in use, but in more recent years the taxonomy of the *Lactobacillus* genus has changed, based on the advent of genomic structure analysis and the further elucidation of phylogenetic relationships between *Lactobacillus* species (Holzapfel et al., 2001). It is now considered that the identification of some *Lactobacillus* species by biochemical methods alone is not reliable (Schleifer et al., 1995).

The identification and typing of lactobacilli to the strain level is vitally important in both an industrial context and for understanding of the diversity of the *Lactobacillus* genus. Many food products and dietary supplements that claim to contain a specific species or strain of *Lactobacillus* may not, in fact, contain that particular organism (Table 1.2) (Holzapfel et al., 2001, Yeung et al., 2002, Mahenthiralingam et al., 2009).

In 1980, six DNA-DNA homology groups were identified, facilitating the definition of *L. acidophilus*, *Lactobacillus crispatus* and *Lactobacillus johnsonii* as type species for three of these groups (Johnson et al., 1980). Above the species level, three groups were initially proposed, consisting of the *Lactobacillus delbrueckii* group, the *Lactobacillus casei*/*Pediococcus* group and the *Leuconostoc* group, which also contained lactobacilli (Collins et al., 1991). In 1995, the *L. delbrueckii* group was reassigned to the *L. acidophilus* group (Schleifer et al., 1995), to better represent its group members, despite *L. delbrueckii* existing as the type species. This study had a polyphasic approach, combining both fermentation characteristics and 16S rRNA gene sequence analysis to define five phylogenetic sub-groups. Since 1995, the genus *Lactobacillus* has undergone numerous taxonomic reconstructions, and at the last review of the taxonomy, the genus *Lactobacillus* (Felis and Dellaglio, 2007) consisted of 14 phylogenetic sub-groups (Table 1.3).

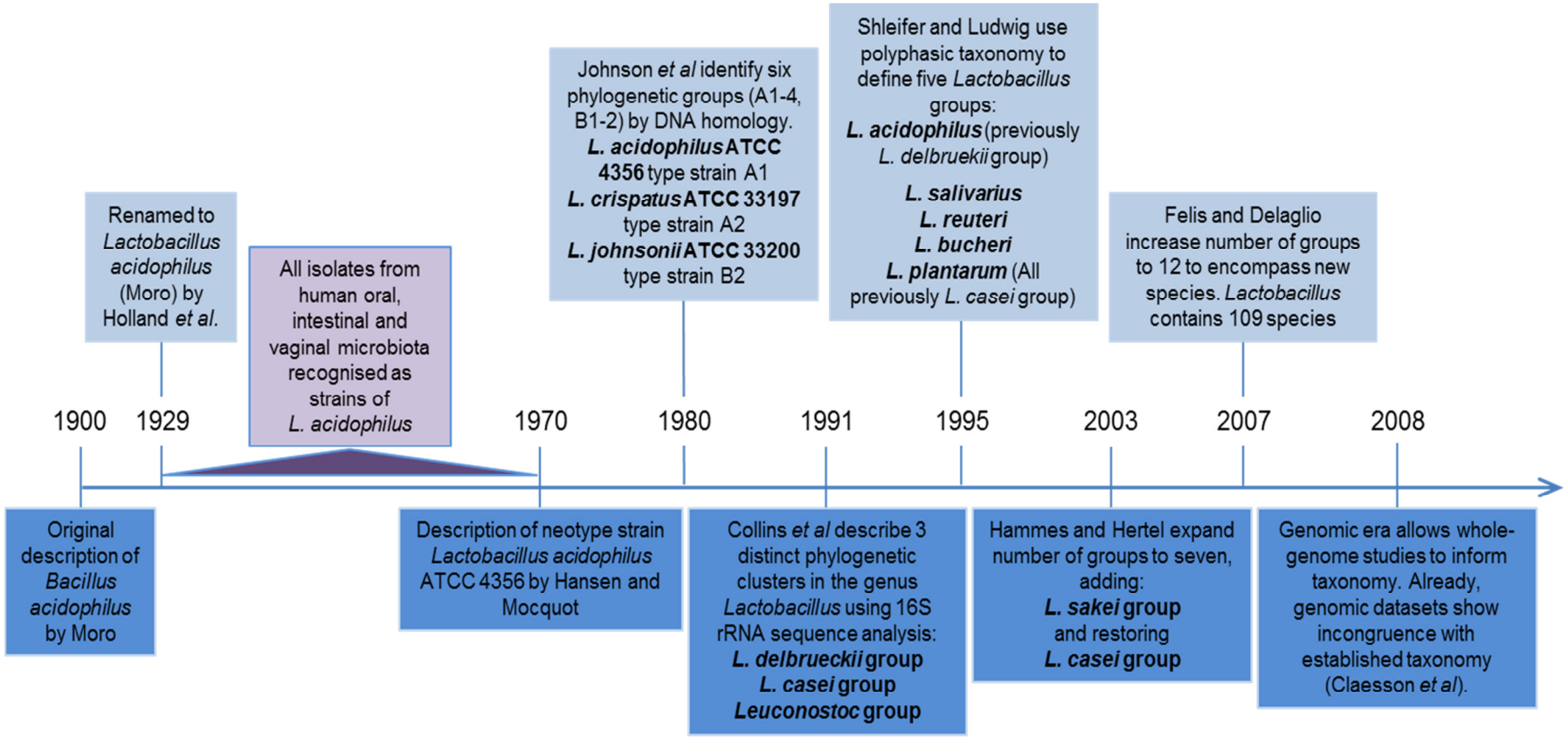


Figure 1.1: History of *L. acidophilus* in the context of the evolving taxonomy of the *Lactobacillus* genus

Major milestones in the development of *Lactobacillus* taxonomy, and the resulting effects on the taxonomic placement of *L. acidophilus*.

Table 1.2: *Lactobacillus* strains used in probiotic yoghurts or yoghurt-like products.

Probiotic strain	Type of product	Identification on the basis of DNA-homology analysis
L. acidophilus LA-1	Yoghurt	*L. johnsonii*
L. acidophilus LA-7	Yoghurt	*L. acidophilus*
L. acidophilus L1	Yoghurt drink	*L. crispatus*
L. acidophilus LA-H3	Dietetic yoghurt	*L. acidophilus*
L. acidophilus	Yoghurt	*L. crispatus*
L. acidophilus	Yoghurt	*L. acidophilus*
L. casei Actimel	Yoghurt drink	*L. paracasei*
L. casei Shirota	Probiotic drink	*L. paracasei*
L. casei GG	Yoghurt drink	*L. rhamnosus*
L. casei LC-H2	Dietetic yoghurt	*L. casei*
L. casei	Yoghurt	*L. paracasei*
L. casei	Yoghurt	*L. paracasei*

Adapted from (Holzapfel et al., 2001)

Table 1.3: Phylogenetic grouping of the genus *Lactobacillus*

L. delbrueckii group (delb)	*L. acetotolerans, L. acidophilus, L. amylolyticus, L. amylophilus, L. amylotrophicus, L. amylovorus, L. crispatus, L. delbrueckii, L. fornicalis, L. gallinarum, L. gasseri, L. hamsteri, L. helveticus, L. iners, L. intestinalis, L. jensenii, L. johnsonii, L. kalixensis, L. kefiranofaciens, L. kitasatonis, L. psittaci, L. sobrius, L. ultunensis*
L. salivarius group (sal)	*L. acidipiscis, L. agilis, L. algidus, L. animalis, L. apodemi, L. aviarius, L. equi, L. mali, L. murinus, L. nageli, L. ruminis, L. saerimneri, L. salivarius, L. satsumensis, L. vini*
L. reuteri group (reu)	*L. antri, L. coleohominis, L. fermentum, L. frumenti, L. gastricus, L. ingluviei, L. mucosae, L. oris, L. panis, L. pontis, L. reuteri, L. secaliphilus, L. vaginalis*
L. buchneri group (buch)	*L. buchneri, L. diolivorans, L. farraginis, L. hilgardii, L. kefiri, L. parabuchneri, L. parafarraginis, L. parakefiri* associated with *L. acidifarinae, L. namurensis, L. spicheri, and L. zymae* (which form a robust group)
L. alimentarius-L. farciminis group (al-far)	*L. alimentarius, L. farciminis, L. kimchii, L. mindensis, L. nantensis, L. paralimentarius, L. tucceti, L. versmoldensis*
L. casei group (cas)	*L. casei, L. paracasei, L. rhamnosus, L. zeae*
L. sakei group (sakei)	*L. curvatus, L. fuchuensis, L. graminis, L. sakei*
L. fructivorans group (fru)	*L. fructivorans, L. homohiochii, L. lindneri, L. sanfranciscensis*
L. coryniformis group (cor)	*L. bifermentans, L. coryniformis, L. rennini*, not robustly associated with *L. composti*
L. plantarum group (plan)	*L. plantarum, L. paraplantarum, L. pentosus*
L. perolens group (per)	*L. perolens, L. harbinensis, L. paracollinoides*
L. brevis group (bre)	*L. brevis, L. hammesii, L. parabrevis*
Pediococcus dextrinicus group (Pdex)	*P. dextrinicus, L. concavus, L. oligofermentans* (the latter sometimes poorly supported)
Pediococcus	2 clusters, not associated: the first comprises *P. cellicola, P. damnosus P. parvulus, P. inopinatus*, while the second includes *P. acidilactici, P. claussenii, P. pentosaceus* and *P. stilesii*
Couples (couple)	(1) *L. rossiae-L. siliginis* (2) *L. vaccinostercus-L. suebicus* (3) *L. manihotivorans-L. collinoides*
Single species (ss)	*L. kunkeei, L. malefermentans, L. pantheris, L. sharpeae, Paralactobacillus selangorensis*

Groups are arranged by descending size. Adapted from (Felis and Dellaglio, 2007).

The *L. acidophilus* group is one of the most well-defined and deep-branching *Lactobacillus* phylogenetic sub-group (Figure 1.2). Although its definition is partially based on DNA-DNA homology, the genomic GC content of constituent species ranges from 32-50% (Felis and Dellaglio, 2007), which is much larger than normally accepted for well-defined bacterial genera (Schleifer and Ludwig, 1995). The dawning of the post-genomic era has now added more tools to the taxonomist's toolkit, providing clarification and as well as further insight into how the taxonomy of the most challenging and complex bacterial groups can be resolved. Recent research into the relatedness of species in the *L. acidophilus* group has used polyphasic taxonomy, combining traditional phenotypic characteristics such as sugar fermentation patterns (Yeung et al., 2004), sequence analyses of genes such as 16S rRNA, *rpoA*, *pheS* (Naser et al., 2007), *groEL* (Claesson et al., 2008), *tuf* (Ventura et al., 2003) and DNA fingerprinting methods such as rep-PCR (Gevers et al., 2001) and Pulsed-Field Gel Electrophoresis (PFGE) (Yeung et al., 2004). Despite highly variable GC content, analyses have shown remarkable congruence with genome microarrays and genomic sequence comparisons, indicating that the *L. acidophilus* phylogenetic sub-group is a natural bacterial group. Genome sequencing now offers a definitive means to identify *Lactobacillus* species and strains (Claesson et al., 2007, Claesson et al., 2008, Salvetti et al., 2012, Bull et al., 2012).

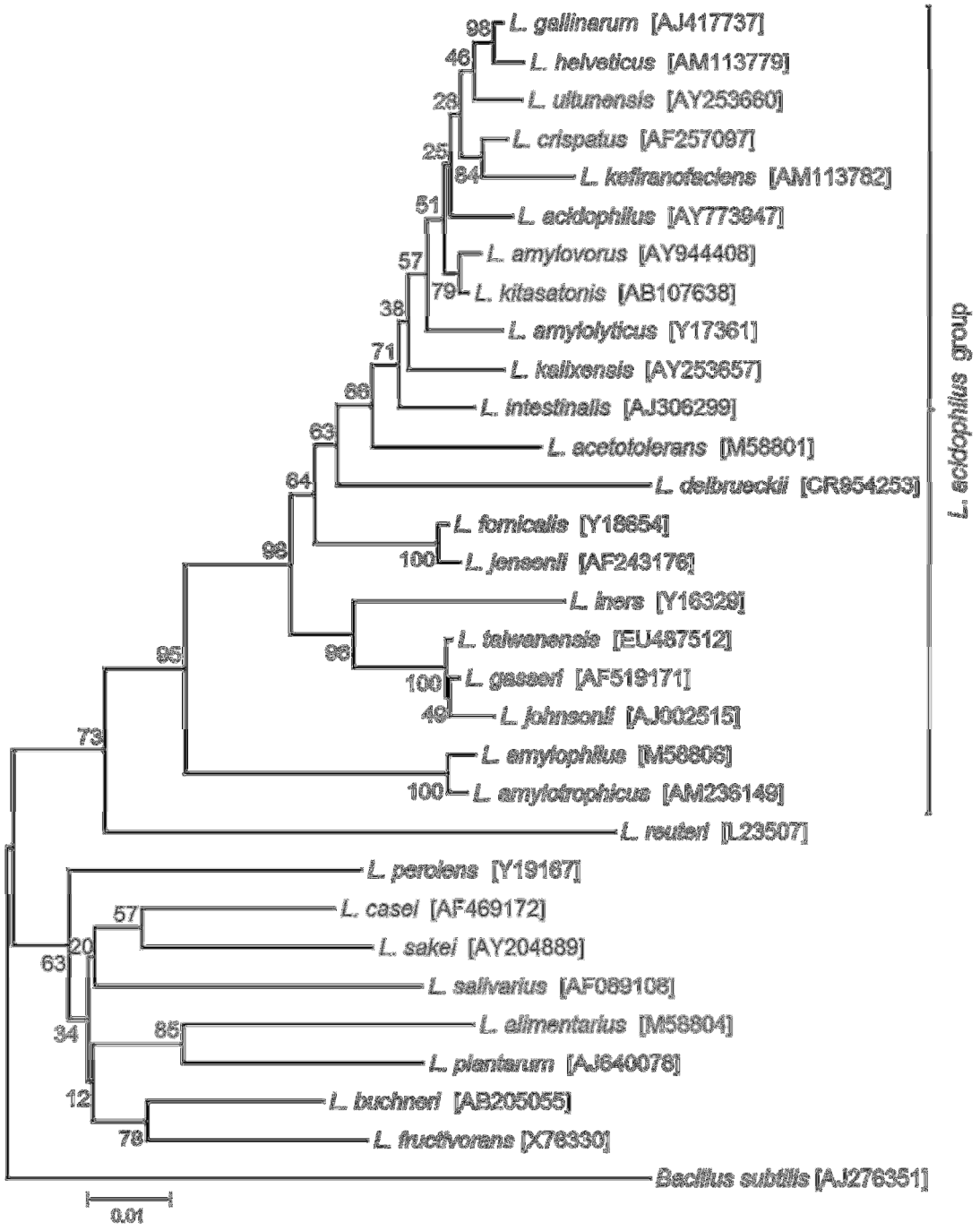


Figure 1.2: Phylogenetic placement of the *L. acidophilus* phylogenetic subgroup within the *Lactobacillus* genus

A phylogenetic tree of aligned 16S rRNA gene sequences from type strains of the *L. acidophilus* phylogenetic sub group (indicated with a brace) and representative type strains from the other *Lactobacillus* phylogenetic sub groups. The tree was rooted with the 16S rRNA gene from *Bacillus subtilis* DSM10. The genetic distance scale, bootstrap values and GenBank nucleotide accession numbers are indicated.

1.3. *LACTOBACILLUS ACIDOPHILUS*

Lactobacillus acidophilus is a LAB species, widely recognised to have probiotic effects and is the most commonly suggested organism for dietary use (Shah, 2007). It is commonly added to yoghurt and fermented milk products, with approximately 80% of the yoghurts produced in the United States in 2006 containing *L. acidophilus* (Sanders, 2003). *L. acidophilus* isolates also form part of the natural human microbiota and have been cultured from oral, digestive and vaginal tracts. By virtue of its widespread use in industry, its probiotic effects and human association, *L. acidophilus* has become one of the most well-studied LAB species. *L. acidophilus* was also the main focus of this PhD study as a bacterial species used by the commercial sponsor of this work (see section 1.6). Key research on *L. acidophilus,* spanning its original isolation from the human gut, through to its genomic and molecular characterization (Figure 1.1) and its major genomic, biosynthetic and probiotic characteristics (Figure 1.3) is summarised below.

1.3.1. *L. ACIDOPHILUS* STRAINS AND THEIR HISTORY

Within the *L. acidophilus* group, there are some 20 species additional to *L. acidophilus sensu stricto* (Table 1.3). It is vital at this point to distinguish between the strain- and species-level classifications of constituent isolates within this group. Much of the early research into the *L. acidophilus* group blurs the lines between bacterial "strains" of the *L. acidophilus* phylogenetic sub-group, which would now be considered as *Lactobacillus* species that belong to the *L. acidophilus* group, and the present definition of a bacterial strain, which is deemed to be a sub-species level taxonomic unit (Klein et al., 1998, Kullen et al., 2000). Considerable research effort has contributed to the revisionary nature of *Lactobacillus* taxonomy, which is important for correctly identifying phylogenetic relationships between species, ensuring species are correctly represented by their type strains, and grouping strains and species in a congruent manner. A lack of rigor and historical understanding of the literature surrounding *L. acidophilus* taxonomy may have also contributed to confusion in identifying the species and strains being studied. The reassignment, for example, of a strain once belonging to *L. acidophilus* (Tuomola and Salminen, 1998) to *Lactobacillus johnsonii*, as an entirely separate species (Pridmore et al.,

2004), had sound systematic support, although some later studies have failed to adopt the correct taxonomic nomenclature (Pimentel et al., 2012).

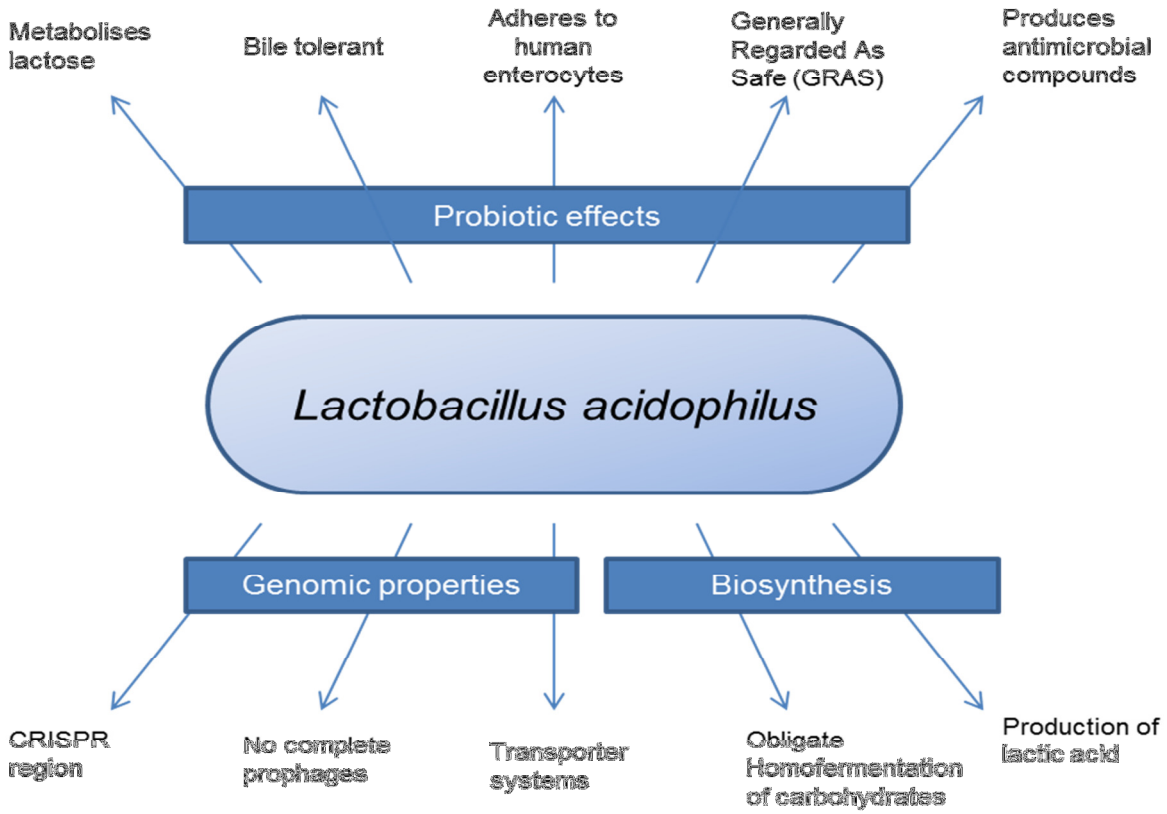


Figure 1.3: Major genomic, biosynthetic and probiotic characteristics of *L. acidophilus*

Historically, *L. acidophilus* has been known for its probiotic effects in humans. Through further characterisation of this effect, and the determination of the genome sequence of *L. acidophilus* NCFM, many biosynthetic capabilities of *L. acidophilus* have been described.

The variety of names that may be attributed to a single strain (Table 1.4), from both culture collections and commercial trademarks, has potentially led to multiple groups unknowingly working with the same strain referred to by a different name (Yeung et al., 2002) and even researchers working on an organism that is not *L. acidophilus* (Bull et al., 2012). The commercial success of *L. acidophilus* may have also contributed to the widespread industrial use of what appear to be identical strains because of their proprietary protection and use within multiple functional foods or probiotic supplements. With this and the taxonomic revisions within the *Lactobacillus* genus in mind, only data/publications related to *L. acidophilus sensu stricto* and strains thereof are collated below.

L. acidophilus was first isolated by in 1900 (Moro) from infant faeces and at the time was described as *Bacillus acidophilus*. As noted above, multiple strain names have been attributed to *L. acidophilus* (Table 1.4). The variety of strain names that have been be given single isolate deposited in multiple locations further complicates establishing the provenance of a particular strain. The StrainInfo database allows users to visually trace the history of a particular strain, and can be used to resolve confusion in many cases (Dawyndt et al., 2005). Fortunately, much of the body of work on *L. acidophilus*, particularly concerning its probiotic effects, has been undertaken on one particular strain: *L. acidophilus* NCFM. Although the depth of information available on NCFM has ensured that it is very well characterised as a true strain of *L. acidophilus*, derived from the neotype strain ATCC 4356 (Sanders and Klaenhammer, 2001), it still has not escaped the confusion of being known by multiple strain names and may exist in the literature as NCFM, N2, NCK56, NCK45 and RL8K (Table 1.4). The large body of information concerning *L. acidophilus* NCFM has contributed to it being deemed Generally Regarded As Safe (GRAS) by the US Food and Drug Administration, as an approved ingredient in dairy products, functional beverages, nutritional powders and more (Bernardeau et al., 2006).

Table 1.4: *L. acidophilus* isolates and their pseudonyms

ATCC[a]	DSMZ[b]	BCCM/LMG[c]	NCIMB[d]	Other Key Names	Notes
ATCC 314		LMG 11467			
ATCC 832		LMG 11428	NCIMB 1723		
ATCC 4355		LMG 11469			
ATCC 4356 T	DSM 20079 T	LMG 13550 T LMG 7943 T LMG 8150 T LMG 9433 T	NCIMB 701748 T NCIMB 8690 T	NCFB 1748 T NCTC 12980 T	Neotype strain (Hansen & Mocquot, 1970)
ATCC 4357	DSM 20242	LMG 11430	NCIMB 8607		
		LMG 13003			
ATCC 4796		LMG 11470			Draft genome sequence (Human Microbiome Project) (Turnbaugh, *et al.*, 2007)
ATCC 9224		LMG 11429 LMG 11472 LMG 19170	NCIMB 8116		
ATCC 13651	DSM 9126	LMG 11466	NCIMB 701360		
ATCC 700396				NCFM, N2, NCK56, NCK45, RL8K	Derived from ATCC 4356 T (Sanders & Klaenhammer, 2001) Genome sequence (Altermann, *et al.*, 2005)

[a] American Type Culture Collection, USA
[b] Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany
[c] Belgian Co-ordinated Collections of Micro-organisms, Belgium
[d] National Collection of Industrial, Food and Marine Bacteria, UK

1.3.2. BASIC FEATURES OF *L. ACIDOPHILUS*

L. acidophilus is a short (2–10 μm) Gram-positive rod that grows optimally from 37°C to 42°C (Altermann et al., 2005) and is able to grow at temperatures as high as 45°C. The species achieves its highest growth rates in slightly acidic media of pH 5.5-6.0, and growth ceases below pH 4.0 (Shah, 2007). It is an obligate homofermenter producing lactic acid from fermentation of carbohydrates and is among the least oxygen tolerant lactobacilli (Archibald and Fridovich, 1981, Claesson et al., 2007).

From examination of the biosynthetic pathways encoded within its genome, *L. acidophilus* is auxotrophic for 14 amino acids and seems unable to synthesise multiple cofactors and vitamins including riboflavin, vitamin B6, nicotinate, nicotinamide, biotin, and folate (Altermann et al., 2005). These deficits in anabolic capacity are exemplified by the need to use nutrient rich media such as deMan, Rogosa and Sharpe (MRS) agar (de Man et al., 1960, Morishita et al., 1981) for its routine culture. *L. acidophilus* forms at least two colony morphotypes when grown under standard culture conditions on MRS agar, referred to as rough and smooth colonies (Figure 1.4). The proportion of rough to smooth colony morphotypes exhibited by *L. acidophilus* is influenced by the exposure to antibiotics such as Penicillin G (Khaleghi et al., 2011) or bile (Khaleghi et al., 2010), which causes a dose-dependent proportional shift towards the smooth morphotype.

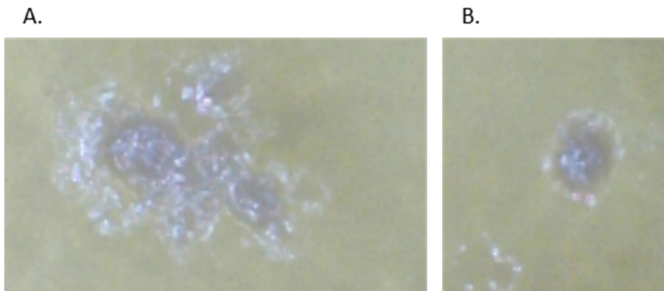


Figure 1.4: Colony morphotypes of *L. acidophilus*

Panel A shows the "rough" colony morphotype, panel B the smooth colony morphotype at 25x magnification on MRS agar after 24h incubation at 37°C

Although *L. acidophilus* has been isolated from multiple human-associated sources (Ahrné et al., 1998, Kulp and Rettger, 1924, Rogosa and Sharpe, 1960) recent phylogenomic characterization by Claesson *et al* (2008) established that the most likely environmental niche of *L. acidophilus* was the GI tract, with other lactobacilli broadly inhabiting plants and meat. The neotype *L. acidophilus* strain ATCC 4356 was described as isolated from the human microbiota, although the records do not give the precise bodily location from where it was isolated.

Metagenomic studies indicate that lactobacilli may compose just 0.2-1% of the total microbiota in the human colon and faeces and also show that their prevalence is highly variable between individuals (Kleerebezem and Vaughan, 2009, Walter, 2008). This suggests that *L. acidophilus* may be just a small and variable fraction of this small carriage of the genus. Culture-independent studies from other hosts also show wide variations in the prevalence of this LAB species. For example *L. acidophilus* was present as the most abundant member of the lactobacilli in broiler chickens (Lu et al., 2003), while in contrast, a total absence of *L. acidophilus* was found in pigs (Leser et al., 2002). Culture-dependent techniques suggest that the lactobacilli in pigs are largely comprised of the *L. acidophilus* group, although no *L. acidophilus* species were isolated (Korhonen et al., 2007). Overall, gut carriage of *L. acidophilus* appears highly variable.

1.3.2.1. Colony-based strain typing and tracking of *L. acidophilus* in the human gut

Human gut passage of *L. acidophilus* has been modelled in a probiotic capsule feeding study (Mahenthiralingam et al., 2009). Participants were pre-screened for faecal presence of *L. acidophilus* using culture-based methods in tandem with DNA fingerprinting to identify the *Lactobacillus* strain being administered. Three of the 12 participants were found to be culture positive for *L. acidophilus* prior to probiotic feeding, indicating faecal carriage of *L. acidophilus* in humans is not universal (Mahenthiralingam et al., 2009). After feeding (5.6×10^9 viable bacteria per capsule, taken daily), the administered *L. acidophilus* strain was detected in 10 out of the 12 subjects, reaching cultivatable levels as

high as 1 x 10^7 colony forming units per gram of faeces in 3 of the volunteers (Mahenthiralingam et al., 2009). Longterm carriage of *L. acidophilus* for 28 days post-feeding was detected in 3 subjects, who notably did not culture *L. acidophilus* before feeding. Overall, these results suggest that dietary intake is a major influence on the human carriage of *L. acidophilus*.

1.3.3. GENOMICS OF *L. ACIDOPHILUS*

The genome sequence of *L. acidophilus* NCFM was the third of the *Lactobacillus* genomes to be published, behind *Lactobacillus plantarum* WCFS1 (Kleerebezem et al., 2003) and *L. johnsonii* NCC 533 (Pridmore et al., 2004); it was the first genome sequence from an *L. acidophilus* phylogenetic sub-group species (Table 1.5). *In silico* analyses of the *L. acidophilus* NCFM shows it is able to synthesise only a limited number of amino acids (cysteine, serine, and aspartate) and to compensate its genome is enriched in genes coding for amino acid transport and fermentative functions (Altermann et al., 2005).

Prophages and horizontally transferred elements are common features of LAB genomes (Foschino et al., 2001). *In silico* analysis of the *L. acidophilus* NCFM genome did not uncover any complete prophages, but three regions constructed from remnants of prophage were discovered due to their constituent ORFs showing homology to phage genes. These three regions were designated as Potential Autonomous Units (PAU) 1-3. All are present within the first 500 ORF of the genome and consist of a core of seven ORF. Further analysis *in silico* predicted a core consisting of an integrase, IntG, a replication protein, RepA, and a DNA segregation ATPase, FtsK, involved in DNA partitioning. The study suggests that PAU3 either evolved in a different organism and was acquired later or was the most ancient integration event into the chromosome. The study also suggested that due to the high degree of similarity between RepA, FtsK, and the two hypothetical proteins flanking FtsK between PAU1 and PAU2 suggested a more recent duplication of PAU1, resulting in PAU2.

Table 1.5: Completed and published genome sequences from the *L. acidophilus* group

Organism	Strain	Origin / Use	GC (mol%)	Genome Size (Mb)	Gene Count	CRISPR Count	Coding Base Count %	Plasmids	Publication
Lactobacillus acidophilus	NCFM	Probiotic	35	1.99	1970	1	89.64	0	(Altermann, *et al.*, 2005)
Lactobacillus amylovorus	GRL 1118	Pig intestine	38	1.98	1994	3	86.86	2	(Kant, *et al.*, 2011)
Lactobacillus amylovorus	GRL 1112	Pig intestine	38	2.13	2193	0	86.99	2	(Kant, *et al.*, 2011)
Lactobacillus crispatus	ST1	Chicken	37	2.04	2100	3	89.37	0	(Ojala, *et al.*, 2010)
Lactobacillus delbrueckii subsp. *bulgaricus*	ATCC 11842	Yoghurt	50	1.86	2234	1	76.01	0	(van de Guchte, *et al.*, 2006)
Lactobacillus delbrueckii subsp. *bulgaricus*	ATCC BAA-365	Cheese, yoghurt	50	1.86	1865	1	79.63	0	(Makarova, *et al.*, 2006)
Lactobacillus delbrueckii subsp. *bulgaricus*	2038	Milk, Probiotic	50	1.87	1907	1	84.52	0	(Hao, *et al.*, 2011)
Lactobacillus delbrueckii subsp. *bulgaricus*	ND02	Milk, Probiotic	50	2.13	2139	2	84.82	1	(Sun, *et al.*, 2011)
Lactobacillus gasseri	ATCC 33323	Human, probiotic	35	1.89	1874	0	90.11	0	(Makarova, *et al.*, 2006)
Lactobacillus helveticus	DPC 4571	Cheese	37	2.08	1830	1	74.8	0	(Callanan, *et al.*, 2008)
Lactobacillus helveticus	R0052	Probiotic	37	2.13	2084	0	80.22	1	(Tompkins, *et al.*, 2012)
Lactobacillus helveticus	H10	Fermented milk	37	2.17	2052	2	81.32	1	(Zhao, *et al.*, 2011)
Lactobacillus johnsonii	DPC 6026	Human	35	1.97	1840	2	88.6	0	(Guinane, *et al.*, 2011)
Lactobacillus johnsonii	FI9785	Human	34	1.79	1804	0	89.64	2	(Wegmann, *et al.*, 2009)
Lactobacillus johnsonii	NCC 533	Probiotic	35	1.99	1941	0	91.09	0	(Pridmore, *et al.*, 2004)
Lactobacillus kefiranofaciens	ZW3	Kefir, Probiotic	37	2.35	2222	3	80.76	2	(Wang, *et al.*, 2011)

The amino acid biosynthetic capability of *L. acidophilus* NCFM is limited, with only cysteine, serine and aspartate capable of being synthesised *de novo*. Subsequently seven derivatives may be synthesised from these three. There were no predicted biosynthesis pathways (*de novo* or by conversion) for the remaining amino acids. This restricted biosynthesis capability is mirrored by the inability of *L. acidophilus* NCFM to synthesise most vitamins and cofactors. This degree of auxotrophy is likely influenced by the nutrient rich environments in which *L. acidophilus* inhabits (e.g. the human gut) and is reflected by its demanding nutrient requirements when grown on synthetic media (Morishita et al., 1981).

The comparatively small (1,993,564 bp) genome of *L. acidophilus* has a low (35%) average GC content, compared to other members of the *L. acidophilus* phylogenetic sub-group (mean GC content = 40%), which have an upper range of 50% GC (*L. delbrueckii* subsp. *bulgaricus*). The GC content of the *L. acidophilus* genome rises to 50% in the four regions containing rRNA genes as expected (Altermann et al., 2005). Other than GC content, basic genomic attributes such as size and gene content do not vary significantly from other member of the *L. acidophilus* group. Plasmids are also common features of members of the *L. acidophilus* group, present in seven of the 16 strains detailed in Table 1.5. Their distribution is heterogeneous, with multiple strains of some species with the same number of plasmids (*L. amylovorus*), some species showing strains with and without plasmids (*L. johnsonii* and *L. helveticus*) and others showing no evidence of plasmids at all (*L. acidophilus* and *L. gasseri*). Despite the lack of *L. acidophilus* NCFM and *L. johnsonii* NCC533 plasmids, a recent study examining phylogenetic trees of 401 proteins identified Horizontal Gene Transfer (HGT) of up to 40% of the core genome genes between the two species, causing an unprecedented level of phylogenetic incongruence (Nicolas et al., 2007).

1.3.4. FOOD AND INDUSTRIAL USE OF *L. ACIDOPHILUS*

L. acidophilus is a major commercial LAB species, present in products including milk, yoghurt and toddler formula, as well as in dietary supplements with reported probiotic effects (Altermann et al., 2005, Sanders and Klaenhammer, 2001). It is part of many undefined starter cultures for milk fermentation, a preservation process that was developed in the Early Neolithic era and has been used in the production of traditional fermented foods for more than 10,000 years (Tamime, 2002). Its slow growth in milk (Azcarate-Peril et al., 2009) means that most of the fermentation in fermented milk products is achieved with a yoghurt starter culture (e.g. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) and then *L. acidophilus* is subsequently added for additional probiotic value (Shah, 2000) in functional milk products.

1.3.5. PROBIOTIC STRAINS OF *L. ACIDOPHILUS*

Probiotic bacterial strains are commonly mislabelled or unlabelled in products, often due to the difficulties in discerning both species and strains of *Lactobacillus* (Yeung et al., 2002). The primary commercial probiotic strains of *L. acidophilus* are described by Shah (2007) and include *L. acidophilus* LA-1 and LA-5 (Chr. Hansen, Denmark), NCFM (Dansico, Madison, US), DDS-1 (Nebraska Cultures, Nebraska, US) and SBT-2026 (Snow Brand Milk Products, Tokyo, Japan). *L. acidophilus* NCFM was developed as a major commercial strain, has identical fermentation and growth characteristics to the type strain ATCC 4356^T and is also closely related in PFGE profile (Sanders and Klaenhammer, 2001). Although similar information regarding the derivation of *L. acidophilus* LA-5 is limited, *L. acidophilus* isolated from products claimed to contain *L. acidophilus* LA-5 produced DNA fingerprints with a high degree of similarity (91.9%) to the type strain *L. acidophilus* ATCC 4356 by Randomly Amplified Polymorphic DNA (RAPD) fingerprint analysis (Schillinger et al., 2003). *L. acidophilus* LA-1 is no longer available as a product from Chr. Hansen . A wealth of research dedicated to "*L. acidophilus* La1" a commercial strain marketed by Nestlé may also be found in the published literature (Link-Amster et al.,

1994). However, this strain has subsequently been taxonomically reassigned to *L. johnsonii* and has a genome sequence available as *L. johnsonii* NCC 533 (Pridmore et al., 2004). Comparative information on the differences in probiotic effect between each commercial strain is not available, however, it is recognised, that different *Lactobacillus* species may display similar probiotic effects *in vitro,* yet have markedly divergent properties when assessed *in vivo* (Ibnou-Zekri et al., 2003).

1.3.6. PROBIOTIC CHARACTERISTICS AND PHYSIOLOGY

The probiotic effects of *L. acidophilus* NCFM are well characterised, aided recently by the availability of its genome sequence and the necessity of in-depth characterisation for application for GRAS status. Although a genome sequence is not available, *L. acidophilus* LA-5 is similarly characterised for patent claim information. The characterisation of probiotic strains may be broadly divided into two categories. The first is desirable probiotic physiology demonstrable *in vitro* such as stability in products (Shah, 2000), resistance to bile (Khaleghi et al., 2010, Pfeiler et al., 2007, Pfeiler and Klaenhammer, 2009) and tolerance to low pH (Azcarate-Peril et al., 2004, Azcarate-Peril et al., 2005), adherence to human enterocytes in cell culture (Buck et al., 2005), antimicrobial production (Sanders and Klaenhammer, 2001, Tabasco et al., 2009) and lactase activity (Sanders et al., 1996). The second category encompasses the gross probiotic effect observable in the context of feeding studies such as mediation of host immune response (Bron et al., 2012), lowering host serum cholesterol (Shah, 2007), improving host lactose metabolism (Gilliland, 1989) and preventing or treating infection (Wang et al., 2004). Several recent feeding trails have also shown that consumption of probiotic products containing L. *acidophilus* NCFM in combination with *Bifidobacterium* species can produce health benefits in the hose, the "gold-standard" for a probiotic label claim. For example, they reduce bloating in adults with functional bowel disorders (Ringel-Kulka et al., 2011) and suppress cold and influenza-like symptoms in children (Leyer et al., 2009).

Analysis of the *L. acidophilus* NCFM genome sequence has directly facilitated the functional characterisation of its ability to tolerate exposure to both low pH and bile, important factors for a probiotic organism that must pass through the gastrointestinal tract. Functional microarray experiments with *L. acidophilus* NCFM showed upregulation of transcripts from three transporter genes (two major facilitator [MFC] superfamily and the permease component of an ABC transporter) in the presence of bile (Pfeiler et al., 2007). Similar transporters had previously been shown in other species to be involved in bile efflux from the cell (Solheim et al., 2007). Furthermore, a study that generated deletion mutants lacking these three transporter genes showed a significant decrease in their ability to survive in bile (Pfeiler and Klaenhammer, 2009). *L. acidophilus* NCFM is also able to survive exposure to pH 3.0 for 5 hours with no loss of viability (Azcarate-Peril et al., 2004), with a two component regulatory system, similar to the *lis*RK system in *Listeria monocytogenes*, implicated in its ability to sense and react to changes in extracellular pH.

L. acidophilus is able to utilise a variety of carbon sources for growth (Sanders and Klaenhammer, 2001, Yeung et al., 2004), but a comprehensive understanding of the mechanisms behind the uptake and metabolism of carbon sources has not yet been achieved. A study describing several genetic loci responsible for carbohydrate metabolism again demonstrated the utility of the *L. acidophilus* complete genome sequence (Barrangou et al., 2006). Several classes of transporter (ATP-binding cassette, phosphoenol-pyruvate phosphotransferase system and galatoside pentose hexuronide permease) were found to be induced in the presence of their respective substrates but repressed in the presence glucose, suggesting that carbohydrate metabolism in *L. acidophilus* is strongly regulated by catabolite repression. The strong link between carbohydrate source and regulation of sugar uptake and metabolism genes likely contributes to the competitive ability of *L. acidophilus* in the human gastrointestinal tract. The metabolism of these complex carbohydrates also provides a function that is not present in humans and other microbiota, potentially enriching the growth of *L. acidophilus* and other probiotic LAB in the human gastrointestinal tract (Zhu et al., 2009).

Studies have demonstrated the ability of *L. acidophilus* to adhere to human Caco-2 enterocytes *in vitro*. An analysis of the adhesion factors involved in *L. acidophilus* NCFM-Caco-2 epithelial cell interaction found significant involvement of S-layer proteins, linked to the gene *slpA*, fibronectin binding protein (FbpA) and mucin binding protein (Mub) (Buck et al., 2005). Alhtough these *in vitro* results suggest that *L. acidophilus* can colonise the gastrointestinal tract, the low dominance seen in metagenomic studies (Kleerebezem and Vaughan, 2009, Walter, 2008), and the lack of persistence seen in probiotic feeding studies (Mahenthiralingam et al., 2009), indicates that *L. acidophilus* may not have primarily evolved as a human gastrointestinal tract organism.

One genomic feature that does vary considerably across *Lactobacillus* genomes are clustered regularly spaced short palindromic repeat (CRISPR) regions. CRISPRs were first described in LAB (Bolotin et al., 2004), are commonly identified in *Lactobacillus* genomes from the *L. acidophilus* phylogenetic subgroup (Table 1.5) and beyond, with approximately half (26/53) of the sequenced *Lactobacillus* genomes possessing CRISPR regions, as identified by BlastP (Koonin and Makarova, 2009). The *L. acidophilus* NCFM CRISPR region has features characteristic of these regions, being approximately 1.5 kb in size and composed of 32 near-perfect 29 base repeats, interspersed with unique 32 base spacer DNAs (Altermann et al., 2005). No physiological function was attributed to CRISPR regions at the time of the NCFM genome publication (Altermann et al., 2005), however subsequent observations that the unique CRISPR spacer sequences were almost identical to fragments of virus and plasmid genes led to the hypothesis that CRISPR regions may be involved in defence against selfish DNA elements (Makarova et al., 2011). This has been validated by the demonstration that a short phage-like sequence inserted into the CRISPR locus of *Streptococcus thermophilus* conferred resistance against its cognate phage (Barrangou et al., 2007).

Prophages and phage interactions are commonly encountered in both the study of LAB genomics and the large scale manufacture of fermented products by LAB (Mahony et al., 2012), where as a result of the economic implications of a large-scale phage contamination

in dairy fermentations, many LAB phages have been well characterised (Brüssow, 2001). The genome sequence of *L. acidophilus* NCFM revealed evidence of three isolated phage remnants, or Potential Autonomous Units (PAUs) designated PauLA-I-III. Each PAU is composed of seven core ORFs, with synteny and ORF size highly conserved between PauLA-I and PauLA-II, with PauLA-III lacking a single ORF of hypothetical function. The high degree of similarity between PauLA-I and PauLA-II suggests that these may have been formed following a duplication event, and PauLA-III was evolved in a different organism and was integrated at a different time to the progenitor or PauLA-I and PauLA-II (Altermann et al., 2005). Interestingly, there is an absence of literature on functional bacteriophages capable of infecting strains of *L. acidophilus sensu stricto* compared to other members of *L. acidophilus* phylogenetic sub-group.

1.3.7. *L. ACIDOPHILUS*: SUMMARY AND PERSPECTIVE

L. acidophilus is an important commercial bacterium with a long history that plays a pivotal role in the characterisation of the genus *Lactobacillus*. However, given the highly progressive nature of *Lactobacillus* taxonomy, *L. acidophilus* as a species has struggled with being misidentified and misrepresented in its past characterisation. Given the increased regulatory criteria being placed on the definition and sale of microbial species as probiotics, *L. acidophilus* strain NCFM has emerged as one of the most well characterized probiotics within this species. However, for other areas of study such as the investigation of environmental niches or microbial composition of fermented foods, care should be taken to clearly identify if *L. acidophilus sensu stricto* strains are present, and going forward it will be important to clarify data provided for both (i) the species level *Lactobacillus* identification, by ensuring new publications are not made with references to old taxonomic names, and (ii) the strain level identification of *L. acidophilus*, by conducting comparisons to well characterised control strains. Ensuring that these parameters are clearly defined for *L. acidophilus* will overcome problems with the multiple strain names used for the same original "isolate" greatly improve our understanding of this biotechnologically important *Lactobacillus* species.

1.4. THE GENUS *BIFIDOBACTERIUM*

All members of the genus *Bifidobacterium* are non-motile, non-sporulating, non-gas-producing and catalase-negative (except *Bifidobacterium indicum* and *Bifidobacterium asteroids*) members of the Actinomycetales branch of the high–G+C Gram-positive bacteria. Generally, Bifidobacteria are anaerobic, although some species can tolerate oxygen (Ventura et al., 2004). All species described so far are grouped in six different ecological niches: the human intestine, oral cavity, the animal gastrointestinal tract (GIT), the insect intestine and sewage, although a faecal contamination event may have caused the rare latter case (De Dea Lindner et al., 2007).

1.4.1. TAXONOMY AND IDENTIFICATION

Bifidobacteria were first isolated from faeces of a breast-fed infant in 1899 (Tissier, 1900), were named *Bacillus bifidus*. Their morphological and physiological features however, which are similar to those of lactobacilli, meant that they were classified as members of the genus *Lactobacillus* for a large part of the 20th century have only been recognized as a different genus relatively recently. The majority of today's species were originally isolated from mammalian GITs (Schell et al., 2002). There are now 38 validly described species belonging to the *Bifidobacterium* genus, with four taxa (*Bifidobacterium animalis*, *Bifidobacterium longum*, *Bifidobacterium pseudolongum* and *Bifidobacterium thermacidophilum*) further divided into subspecies, all of which display greater than 93% identity of their 16S rRNA gene sequences (Miyake et al., 1998) (Figure 1.5).

Traditional identification and typing of bifidobacteria was similar to lactobacilli, with the methods relying on examining phenotypic characteristics to differentiate species. Enzymatic and carbohydrate acidification profiles were instrumental in characterising new isolates and redefining clusters of species (Bahaka et al., 1993). These carbohydrate fermentation patterns have been shown to be more related to strains than species however (Roy and Ward, 1990, Roy et al., 1996), and more appropriate methods have since been applied.

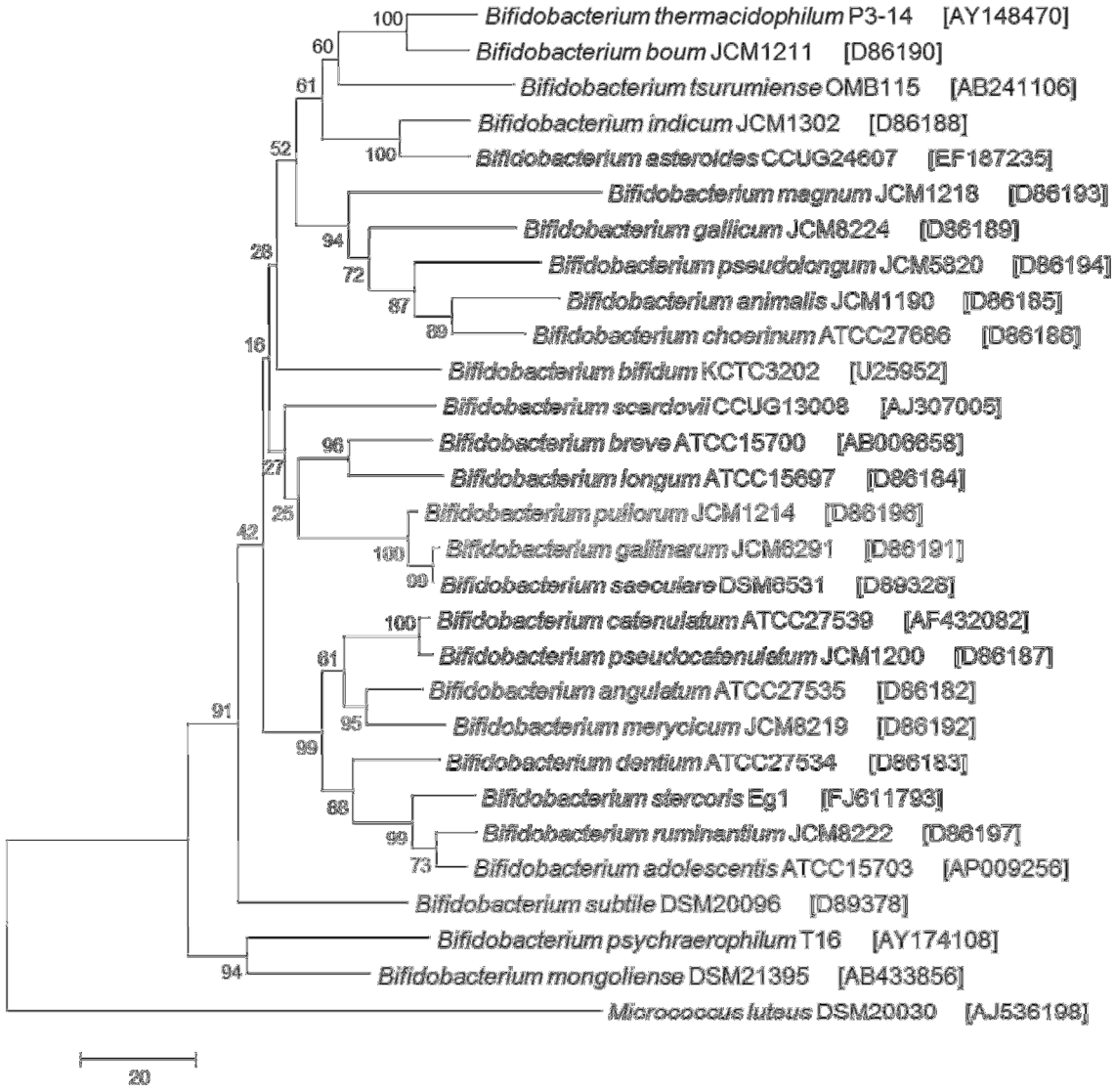


Figure 1.5: Phylogenetic relationships within the *Bifidobacterium* genus

A phylogenetic tree of aligned 16S rRNA gene sequences from available type strains of the *Bifidobacterium genus*. The tree was rooted with the 16S rRNA gene from *Micrococcus luteus* CSM 20030. The genetic distance, scale, bootstrap values and GenBank nucleotide accession numbers are indicated.

16S rRNA gene sequence homology analysis has proved a powerful method for analysing phylogenetic relationships between other bacteria (Stackebrandt and Goebel, 1994). *Bifidobacterium* 16S rRNA gene sequences showed only 7% difference across the whole genus (Zhu et al., 2003), with four groups of species showing just 1% sequence divergence across multiple species (Miyake et al., 1998), too similar to differentiate between these species. DNA-DNA hybridisation was subsequently evaluated as a superior method for speciating closely related bifidobacterial species. Other conserved macromolecules such as the *tuf* gene encoding the Tu elongation factor, *rec*A encoding recombinase A and *gro*EL gene have all been proposed as alternative molecular chronometers for bifidobacteria (Ventura et al., 2004).

Molecular fingerprinting methods such as RAPD (Vincent et al., 1998, Simpson et al., 2003) and PFGE using *Xba*I (McCartney et al., 1996, Simpson et al., 2003) have been used successfully to describe both intra- and inter-species diversity of bifidobacteria. In the current era of relatively inexpensive and highly accurate genome resequencing however, the next "gold-standard" of isolate identification and elucidation of taxonomic and phylogenetic relationships is the complete genome sequence of an isolate.

1.4.2. *BIFIDOBACTERIUM* GENOMICS

Currently, the genus *Bifidobacterium* is represented by 38 species, of which 10 have at least one isolate with its genome completely sequenced and publically available (Table 1.6). This is in direct contrast to the *Lactobacillus* genus whose widespread use as active ingredients in functional foods, and their extreme phylogenetic, phenotypic and ecological diversity has ensured many more representative genomes have been decoded (Ventura et al., 2009a). The generation and analysis of bifidobacterial genome sequences has forged the discovery and analysis of genetic and metabolic characteristics of these important probiotic bacteria, alongside enhancing knowledge of their evolutionary relationships.

1.4.2.1. Comparative genomics

The chromosomes of bifidobacteria range in size from 1.9 to 2.8 Mb, with a GC content of between 59 and 63% (Table 1.6). This is again in direct contrast to the genomes of lactobacilli, which have much greater diversity of both size (Ventura et al., 2012) and GC content (Table 1.5). It should be pointed out however, that bifidobacteria with reduced genomes are extensively used in functional foods, and therefore may have been propagated for a considerable length of evolutionary time in synthetic media. It has been demonstrated that such treatment of bifidobacteria may cause genome decay (Lee et al., 2008), with isolates losing apparently dispensable regions of their chromosomes in an environment different from their original ecological niche.

Extrachromosomal elements in the form of plasmids are common amongst all bacteria. *B. longum* strains show some diversity in the number of plasmids harboured (Table 1.6), with *B. longum* subsp. *longum* DJO10A harbouring two plasmids, pDOJH10L (10 kb) and pDOJH10S (3.6 kb) (Bottacini et al., 2010), while *B. longum* subsp. *longum* NCC2705 possesses a single plasmid, pBLO1 (3.6 kb) (Schell et al., 2002). All other sequenced genomes appear to lack plasmids. There are no obvious unusual species-specific features with regard to the coding density.

A comparison of genomes across the whole *Bifidobacterium* genus showed that the bifidobacterial pan-genome was likely to consist of more than 5000 genes (Bottacini et al., 2010), more than double the coding capacity of a single bifidobacterial genome. The core genome shared by all bifidobacterial isolates was 967 genes, with mostly housekeeping function (replication, transcription, translation, cell envelope biosynthesis and signal transduction) (Bottacini et al., 2010). Genes unique to each genome ranged from 21 to 230 across the nine genomes studied, with many of unknown function (Bottacini et al., 2010), although given the propensity of bifidobacteria to dispense of genomic regions not useful to their present lifestyle and niche, they are likely to have a specialized role within that particular niche (Turroni et al., 2011).

Table 1.6: Completed *Bifidobacterium* genome sequences

Genome Name / Sample Name	Strain	Origin / Use	GC %	Genome Size (Mb)	Gene Count	Coding Base Count (%)	Plasmids	Publication
Bifidobacterium adolescentis	ATCC 15703	Human, probiotic	59	2.1	1709	87.83	0	NCBI RefSeq
Bifidobacterium animalis subsp. *animalis*	ATCC 25527	Rat	60	1.9	1597	85.88	0	(Loquasto et al., 2011)
Bifidobacterium animalis subsp. *lactis*	Bl-04	Human, probiotic	60	1.9	1631	87.15	0	(Barrangou et al., 2009)
Bifidobacterium animalis subsp. *lactis*	AD011	Human, probiotic	60	1.9	1587	85.13	0	(Kim et al., 2009)
Bifidobacterium animalis subsp. *lactis*	DSM 10140	Human, yoghurt, probiotic	60	1.9	1629	86.94	0	(Barrangou et al., 2009)
Bifidobacterium animalis subsp. *lactis*	BB-12	Probiotic	60	1.9	1706	90.39	0	(Garrigues et al., 2010)
Bifidobacterium animalis subsp. *lactis*	CNCM I-2494	Human, probiotc	60	1.9	1724	90.86	0	(Chervaux et al., 2011)
Bifidobacterium animalis subsp. *lactis*	B420	Probiotic	60	1.9	1625	86.03	0	(Stahl and Barrangou, 2012)
Bifidobacterium animalis subsp. *lactis*	V9	Human, probiotic	60	1.9	1636	87.20	0	(Sun et al., 2010)
Bifidobacterium animalis subsp. *lactis*	BLC1	N/D	60	1.9	1622	87.54	0	(Bottacini et al., 2011)
Bifidobacterium animalis subsp. *lactis*	Bi-07	Probiotic	60	1.9	1625	86.03	0	(Stahl and Barrangou, 2012)
Bifidobacterium asteroides	PRL2011	Honey bee GIT	60	2.2	1731	87.44	0	(Bottacini et al., 2012)
Bifidobacterium bifidum	BGN4	Human, probiotic	63	2.2	1903	86.12	0	(Yu et al., 2012)
Bifidobacterium bifidum	S17	Human, probiotic	63	2.2	1845	86.04	0	(Zhurina et al., 2011)
Bifidobacterium bifidum	PRL2010	Human, probiotic	63	2.2	1767	84.40	0	(Turroni et al., 2010)
Bifidobacterium breve	UCC2003	Human, probiotic	59	2.4	1914	84.63	0	(O'Connell Motherway et al., 2011)
Bifidobacterium breve	ACS-071-V-Sch8b	Human, vaginal	59	2.3	1890	83.77	0	Human Microbiome Project

N/D: No Data

Table 1.6: Completed Bifidobacterium genome sequences (cont.)

Genome Name / Sample Name	Strain	Origin / Use	GC (mol%)	Genome Size (Mb)	Gene Count	Coding Base Count (%)	Plasmids	Publication
Bifidobacterium dentium	Bd1	Human, dental, probiotic	59	2.6	2197	86.95	0	(Ventura et al., 2009b)
Bifidobacterium longum	DJO10A	Human, probiotic	60	2.4	2074	87.14	2	(Lee et al., 2008)
Bifidobacterium longum	NCC2705	Human, probiotic	60	2.3	1805	86.32	1	(Schell et al., 2002)
Bifidobacterium longum subsp. *infantis*	157F	Human, commensal	60	2.4	2070	86.73	2	(Fukuda et al., 2011)
Bifidobacterium longum subsp. *infantis*	ATCC 15697	Human, probiotic	60	2.8	2577	85.88	0	(Sela et al., 2008)
Bifidobacterium longum subsp. *infantis*	JCM 1222	Human, commensal	60	2.8	2641	86.17	0	(Fukuda et al., 2011)
Bifidobacterium longum subsp. *longum*	KACC 91563	Biotechnological	60	2.4	2050	86.56	2	(Ham et al., 2011)
Bifidobacterium longum subsp. *longum*	JDM301	Human	60	2.5	2022	85.60	0	(Wei et al., 2010)
Bifidobacterium longum subsp. *longum*	BBMN68	Human	60	2.3	1870	85.43	0	(Hao et al., 2011b)
Bifidobacterium longum subsp. *longum*	JCM 1217	Human, commensal	60	2.4	2009	87.03	0	(Fukuda et al., 2011)
Bifidobacterium longum subsp. *longum*	F8	Human, commensal	60	2.4	1744	72.09	0	MetaHIT

1.4.3. PHYSIOLOGICAL AND PROBIOTIC CHARACTERISTICS

1.4.3.1. Carbohydrate metabolism

Carbohydrate metabolism is particularly instrumental in the life of Bifidobacteria in the mammalian GIT, with the availability of many complex polysaccharides (e.g. xylose and arabinose containing compounds) that are beyond the hydrolysing capability of host enzymes, which are mostly restricted to disaccharides (e.g. lactose and sucrose) and specific polysaccharides such as starch (Ventura et al., 2007b). These more complex polysaccharides would be unavailable to the host if the diverse mixture of anaerobic bacteria (including bifidobacteria) were not present in the gut. This is a mutually beneficial relationship, were the host gains carbon and energy through short-chain fatty acid absorption, and the bacterial community have access to a wide variety of glycans in a protected anoxic environment (Ventura et al., 2007b). For context, bacteria such as *Lactobacillus* tend to be more numerous in the upper GIT (Vaughan et al., 2005) where they ferment relatively simple mono- and di-saccharides, and bifidobacteria more common in the lower GIT where their success is likely due to their capacity to metabolise the complex carbohydrates that are more common in the large intestine.

Bifidobacterial genomes contain genes that reflect their adaptation to the GIT niche, exemplified by the large number of genes encoding carbohydrate-modifying enzymes, such as glycosyl hydrolases, sugar ABC transporters, and PEP-PTS (PEP—phosphoenolpyruvate; PTS—phosphotransferase system) components, all of which are needed for the metabolism of carbohydrates (Barrangou et al., 2009, Kim et al., 2009, Schell et al., 2002). Additionally a large number of genes are predicted to be involved in sugar metabolism, with almost half of these are devoted to carbohydrate uptake, by means of ABC transporters, permeases and proton symporters, rather than through PEP-PTS transport (Ventura et al., 2012).

Hexose sugars are metabolised through a metabolic pathway unique to bifidobacteria, referred to as the “bifid-shunt”, which is built around the fructose-6-phosphoketolase

enzyme (F6PPK.) (de Vries and Stouthamer, 1967). This enzyme is considered to be a taxonomic marker for the family of *Bifidobacteriaceae* (Felis and Dellaglio, 2007). When operated in tandem with enzymes for transporting carbohydrates, encoded in the rest of the bifidobacterial genome, the bifid-shunt confers a competitive advantage on *Bifidobacteria* by producing more ATP from the same quantity of carbohydrate than fermentative pathways, like those operating in homofermentative LAB for example (Pokusaeva et al., 2011).

1.4.3.2. Temperature and oxygen requirements

Bifidobacteria display niche specific growth temperature requirements, with isolates from humans GIT able to grow at temperatures ranging between 36°C and 38°C. In contrast, *Bifidobacterium* species isolated from animals GITs show growth at higher temperatures (41°C to 43°C), with *B. thermacidophilum* exhibiting a maximal growth temperature of 49.5 °C (Ventura et al., 2004), showing general adaptations to temperature conditions in their respective niches.

Bifidobacteria are described as strict anaerobes although some species (*B. lactis*, *B. aerophilum* and *B. psychroaerophilum*) can tolerate oxygen (Ventura et al., 2004). The biochemistry of oxygen requirements is different for each bifidobacterial species, with weak catalase activity or the presence of NADH oxidase able to confer oxygen tolerance, by removing or avoiding the synthesis of hydrogen peroxide (de Vries and Stouthamer, 1968). In species that are highly sensitive to oxygen, accumulation of hydrogen peroxide is the principal reason for reduced metabolism since its presence inactivates F6PPK, a key enzyme of the “Bifid-shunt” (Ventura et al., 2004).

1.4.3.3. Probiotic effect and host interactions

There is economical interest in the probiotic effect of bifidobacterial isolates, and numerous strains are added in high numbers (commonly greater than 10^9 cfu per dose) to products to create functional foods (Stanton et al., 2005). When applied to the human GIT, probiotic microorganisms have a potentially symbiotic relationship with their host, contributing to host nutrition by impacting on intestinal cell proliferation and differentiation, modulating the host immune system, competitively excluding pathogenic microorganisms and supressing intestinal inflammation (Saxelin et al., 2005). In the bifidobacterial context, probiotic effect is built on the foundation of carbohydrate metabolism combined with the highly efficient bifid-shunt pathway. The complex carbohydrates that are not able to be digested by the endogenous, host enzymatic suite (including fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), gluco-oligosaccharides, xylo-oligosaccharides, inulin, starch, arabinoxylan and arabinogalactan, lactulose and raffinose (Guarner and Malagelada, 2003, Turroni et al., 2011)) constitute fermentable substrates for intestinal bacteria such as *Bifidobacterium*.

In neonates, human milk is often the only source of nutrition. It contains Human Milk Oligosaccharides (HMO), complex oligosaccharides that are central carbohydrate constituents of this nutrient-rich food (Asakuma et al., 2008). These complex oligosaccharides are built from N-acetylglucosamine, D-glucose, D-galactose, L-fucose and N-acetylneuraminic acid residues in a large variety of carbohydrate configurations (Sela and Mills, 2010). Bifidobacteria and other early colonisers of the human gut are capable of degrading HMOs and isolates of *B. bifidum* and *B. longum* from infants are typically do so, although isolates recovered from adults are usually not able to utilise HMOs (Sela and Mills, 2010), potentially due to genome reduction of non-essential genes.

Bifidobacterial genome sequences all appear encode extracellular polysaccharide (EPS), which may be implicated in adherence to host cells in the GIT and potentially confers increased levels of resilience to stomach acids and bile salts (Perez et al., 1998, Ventura et al., 2007a), which is important in a probiotic context and especially so if the probiotic is

fed in a "live culture" that must traverse the entirety of the GIT. Additionally, the genomes of both lower-GIT (Klijn et al., 2005) and oral (Ventura et al., 2009b) originating bifidobacteria are predicted to encode glycoprotein fimbriae-like structures, which are implicated in microbial adhesion and colonization of host epithelial cell surfaces in other, well-studied, probiotic bacteria (Kankainen et al., 2009).

1.4.4. *BIFIDOBACTERIUM ANIMALIS* SUBSP. *LACTIS*

1.4.4.1. Characteristics and physiology

Bifidobacterium animalis subsp. *lactis* is commonly found in the GIT of healthy humans and the infant gut microbiota, primarily in faecal (Turroni et al., 2009) and ileal (Wall et al., 2007) samples. It is also the most common *Bifidobacterium* species used as a probiotic in commercial dairy products in both North America and Europe (Gueimonde et al., 2004, Masco et al., 2005). By virtue of its wide use and commercial probiotic importance, many studies have been undertaken using strains of *B. animalis* subsp. *lactis* to elucidate their potentially probiotic effects. Potential probiotic effects include survival in the GIT (Wall et al., 2007), modification of host fecal flora (Bartosch et al., 2005), modulation of the host immune response (Paineau et al., 2008), adherance to human epithelial cells *in vitro* (Gopal et al., 2001) and prevention of microbial gastroenteritis and colitis (Philippe et al., 2011). In addition to the above specific probiotic properties, *B. animalis* subsp. *lactis* retains the ability of the rest of the genus to metabolise oligosaccharides that are not digestible by the host, contributing to its ability to compete in the human gut (Barrangou et al., 2009). The described benefits linked with certain strains of *B. animalis* subsp. *lactis* have resulted in their inclusion in a large array of dietary supplements and foods (see Table 1.1), including dairy products such as yoghurt.

Despite the commercial and probiotic significance of *B. animalis* subsp. *lactis* however, methods for differentiating *B. animalis* subsp. *lactis* at the strain-level has proved challenging, due to the genetic identity of the species as determined by PFGE (Briczinski and Roberts, 2006) and other nucleic acid-based techniques such as ERIC-PCR (Ventura

and Zink, 2002a). Currently, eight *B. animalis* subsp. *lactis* genome sequences are complete and publically available (Table 1.6), providing both reference sequences for ongoing metagenomic analyses of the human environment, and a detailed insight into the strain-level diversity of *B. animalis* subsp. *lactis*.

1.4.4.2. Strain-level diversity

A recent study by Milani *et al* (2013) used comparative genome sequence analysis to establish the strain-level diversity of *B. animalis* subsp. *lactis* at the genomic level. Very high genome sequence similarity was observed between strains of *B. animalis* subsp. *lactis*, corroborated by a high degree of conservation in terms of genome size, organization, and sequence (Milani et al., 2013). It was postulated that the isogenic nature of all *B. animalis* subsp. *lactis* strains, combined with the overall absence of polymorphism was symptomatic of a genetically monomorphic subspecies. A pan-genome analysis of the *B. animalis* subsp. *lactis* taxon indicated a closed pan-genome, suggesting no new genetic coding ability will be discovered by additional genomic resequencing attempts (Milani et al., 2013). This signifies that *B. animalis* subsp. *lactis* is a highly clonal, potentially recently evolved, taxon from the *B. animalis* species, although alternatively, the resequenced strains may all be members of the same clade and the genetic diversity of *B. animalis* subsp. *lactis* may not be adequately represented within these strains. The only validated difference between strains of *B. animalis* subsp. *lactis* was found in the non-coding CRISPR region (Section 1.3.3), where three homology groups were observed with 19, 20 and 23 CRISPR repeats respectively (Milani et al., 2013).

1.4.4.3. Strain typing

Briczinski *et al* (2009) proposed a method for differentiating between strains of *B. animalis* subsp. *lactis* using Single Nucleotide Polymorphisms (SNPs), insertions and deletions. 24 strains of *B. animalis* subsp. *lactis* were indistinguishable by both PFGE and RAPD using seven arbitrary primer sets (Briczinski et al., 2009). Strain-specific differences in sugar fermentation patterns were observed, although differences depended on the original culture medium for each isolate, with the exception of glucose uptake, whose rate correlated with a

SNP in the glucose transporter *glcU* (Briczinski et al., 2009). In the 50 variable genetic loci (SNPs), transporters and CRISPR elements were highly represented, perhaps indicating selective pressure on hypervariable loci (CRISPR) and genes involved in adaptation to an environmental niche (transcriptional regulators, carbohydrate uptake and metabolism) (Briczinski et al., 2009). The type strain, *B. animalis* subsp. *lactis* DSMZ 10140, was the most genetically distinct from the rest of the species, with other isolates falling into distinct families of strains, broadly correlating with the single SNP in *glcU* and therefore rate of glucose metabolism (Briczinski et al., 2009).

1.4.5. *BIFIDOBACTERIUM BIFIDUM*

1.4.5.1. Probiotic Characteristics

Bifidobacterium bifidum is one of the four major bifidobacterial species commonly detected in adult and infant faeces (Matsuki et al., 2003). Healthy infants were found to have proportionally high levels of faecal *B. bifidum* compared to infants that displayed raised immunoglobulin E (IgE) responses to common environmental antigens, indicating that they were allergic (He et al., 2001). When fed in combination with antibiotics, *B. bifidum* OFR9 and prevented an overall decrease in the numbers of bifidobacteria in the gut, and restored the gut microbiota to normal more rapidly than just antibiotic (Chung et al., 1997). Other potential probiotic characteristics of strains of *B. bifidum* include modulation of the host immune response (Ko et al., 1999, Park et al., 2002), production of bifidocin B, a bacteriocin (Yildirim et al., 1999). *B. bifidum* PRL2010 is also able utilise host-derived glycans such as mucin (Turroni et al., 2010).

1.5. EXAMINING STRAIN-LEVEL DIVERSITY

Strain typing of bacteria is based on the assumption that the clonal descendants of a single ancestor will share characteristics that will differ from those of unrelated strains. These characteristics should be diverse within the species yet both stable and easily measurable. Many common bacterial typing methods have been adapted to encompass the typing of lactobacilli. The typing methods detailed below require the prior cultivation of isolates to be typed, in order to obtain either separate colonies or pure cultures (Dykes and von Holy, 1994).

1.5.1. DNA FINGERPRINTING METHODS

1.5.1.1. Pulsed Field Gel Electrophoresis (PFGE)

PFGE allows the high-resolution separation of DNA fragments of a larger size than permitted in traditional gel electrophoresis with the use of a continually reorienting (or pulsed) electric field. In PFGE, the complete bacterial genome is digested with a sparsely-cutting restriction enzyme (such as *Not*I or *Sfi*I for lactobacilli (Tynkkynen et al., 1999)) and then subjected to pulsed-field gel electrophoresis in order to separate the fragments of DNA. Tynkkynen et al. (1999) suggest that PFGE is the most discriminatory method for typing isolates of *Lactobacillus rhamnosus* and *Lactobacillus casei*.

1.5.1.2. RAPD Fingerprinting

RAPD fingerprinting uses short (usually ~10 bp) oligonucleotides with a random sequence as a primer in a low stringency PCR. The primers anneal to complementary or partially complementary regions on the target DNA. When the primers anneal on opposite strands within approximately 1 kb of one another, a PCR amplicon is generated. The PCR product is then subjected to separation by size by electrophoresis, and a RAPD fingerprint is created. This method generally allows differentiation between species and to some extent, strains within a species (Satokari et al., 2003). RAPD fingerprinting has been widely applied to the LAB (Du Plessis and Dicks, 1995). RAPD fingerprinting is generally

considered to be simple and rapid, although care needs to be taken to ensure the reproducibility of results generated by this PCR-based method.

1.5.1.3. Repetitive Element PCR Fingerprinting

Rep-PCR genomic fingerprinting makes use of DNA primers complementary to naturally occurring, highly conserved, repetitive DNA sequences, present in multiple copies in the genomes of several Gram-positive bacteria, including lactobacilli (Stephenson et al., 2009). There are three families of repetitive sequences; the 35-40 bp repetitive extragenic palindromic (REP) sequence, the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence (de Bruijn, 1992), and the 154 bp BOX element (Gevers et al., 2001). These sequences are well distributed in intergenic positions around the genome. PCR primers have been designed to allow DNA synthesis outward from the inverted repeats in REP and ERIC, and from the boxA subunit of BOX. This PCR leads to the amplification of distinct genomic regions located between REP, ERIC or BOX elements. The corresponding procedures are referred to as REP-PCR, ERIC-PCR and BOX-PCR genomic fingerprinting respectively. The PCR amplicons may then be resolved using agarose gel electrophoresis, yielding a rep-PCR genomic fingerprint. REP-PCR and ERIC-PCR have been applied to lactobacilli, and have shown excellent discriminatory ability at the subspecies level (Gevers et al., 2001, Ventura and Zink, 2002b).

1.5.2. DNA SEQUENCE-BASED METHODS

1.5.2.1. Multi Locus Sequence Typing (MLST)

MLST was developed by Maiden *et al* (1998) for typing *Neiserria meningitidis* and has been effectively applied to strain typing of bacterial pathogens such as *Escherichia coli* (Lacher et al., 2007) and the *Burkholderia cepacia* complex (Baldwin et al., 2005). MLST schemes for species of LAB, including *Lactobacillus plantarum* (de las Rivas et al., 2006) and *L. casei* (Cai et al., 2007) have also been developed. MLST relies on partially sequencing six or seven 'housekeeping' genes, allowing the characterisation of alleles at

relatively conserved genomic loci, and as such, may differentiate between bacterial strains with much greater resolution than rRNA gene sequence analysis, which relies on polymorphism at a single locus. Each unique sequence type at each locus is designated a number, and hence strains may be defined by their seven-number sequence type. The MLST scheme for *L. casei* targets the genes encoding the following proteins: protein elongation factor EF-2 (*fusA*), isoleucyl-tRNA synthetase (*ileS*), GTP-binding protein LepA (*lepA*), leucyl-tRNA synthetase (*leuS*), CTP synthetase (*pyrG*), recombinase A (*recA*) and ATP-dependent DNA helicase (*recG*). The *L. plantarum* MLST scheme targets the genes encoding the following proteins: phosphoglucomutase (*pgm*), D-alanine-D-alanine ligase (*ddl*), B subunit of DNA gyrase (*gyrB*), ATPase subunit of phosphoribosylaminoimidazole carboxylase (*purK1*), glutamate dehydrogenase (*gdh*), DNA mismatch repair protein (*mutS*) and transketolase (*tkt4*).

1.5.3. SPECIES SPECIFIC PROBES BASED ON RIBOSOMAL RNA SEQUENCES

Numerous specific oligonucleotide probes, specifically targeting rRNA genes, have been designed for different species of human intestine occurring lactobacilli (Satokari et al., 2003). These are designed by analysing the sequence of rRNA genes to discover variable regions that contain information suggestive of phylogenetic groupings of different levels. Probes may therefore be designed to detect and differentiate organisms at the group, genus, species or strain level, providing there is sufficient sequence diversity at each level. Lactobacilli present a challenge when designing probes that differentiate at the genus level or below because of their phylogenetic heterogeneity.

The development of species specific probes allows the detection of specific DNA sequences by PCR. This, in turn, allows quantification of these sequences in the environment, providing information concerning the quantity of the organism to which the sequence is specific. Number of sequences present and numbers of viable organisms are not directly equivalent however, as most species possess more than one copy of the rRNA gene sequence in question, and the presence of a specific sequence does not indicate a viable organism.

1.5.4. THE POST-GENOMIC ERA

1.5.4.1. Comparative genomics

The advent of relatively cheap and highly accurate next-generation sequencing technologies has made the reseqeuncing of bacterial strains a viable laboratory method for strain typing, which also provides a vast amount of information supplementary to strain type. The decoding of an isolate's genome sequence is the ultimate genotyping method (Li et al., 2009). The progressive gathering of genotyping information from DNA fingerprinting methods to whole genome sequencing has driven improvements in taxonomical accuracy across all bacterial species; pathogens, industrial organisms and probiotics. Relevant examples of the use of comparative genomics in the wider context of this study are provided for the lactobacilli (Section 1.2.2) and *B. animalis* subsp. *lactis* (Section 1.4.4.2). Recently, the development of ribosomal MLST (rMLST) provides resolution from the sub-species level to the whole bacterial domain, by indexing variation of sequences that encode ribosomal proteins (*rps* genes) (Jolley et al., 2012), and is discussed in more detail in Chapters 4 and 6.

1.6. PROJECT AIMS

Probiotic bacteria are a diverse and heterogeneous group of organisms that have been utilised since the turn of the century for their health benefits and for thousands of years in fermented dairy and other products. Comparatively little is known about their population biology and evolution, specific mode of action, and their effects on both the metabolites and indigenous microbiota of the human gut.

This PhD, a Collaborative Awards in Science and Engineering (CASE) studentship was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) in collaborative partnerhip with Cultech Ltd., an internationally recognised innovator and premium quality manufacturer within the nutritional supplement industry, based in Port Talbot, Wales, UK. The questions and aims investigated in this study reflect their commercial interests, characterising and examining the diversity of probiotic bacteria at the molecular level, with specific focus on isolates used by Cultech Ltd. To bring a genetic context to the phenotypic information available on the bacteria used by Cultech Ltd., state-of-the-art next-generation sequencing combined with genome-scale analysis techniques was used to build upon classical strain typing methods.

Cultech Ltd. produce four different nutritional supplements, three for the UK and one for the North American markets, all of which contain the Lab4® probiotic mixture. Lab4® consists of two strains of *L. acidophilus*, CUL 21 and CUL 60 and two bifidobacterial strains *B. bifidum* CUL 20 and *B. animalis* subsp. *lactis* CUL 34. Clinical trials conducted with the Lab4® probiotic mixture have been shown prevent atopic sensitization and atopic eczema when administered to pregnant women and infants aged 0-6 months (Allen et al., 2012), significantly reduce total symptoms and improve quality of life in diagnosed IBS sufferers (Williams et al., 2009) and reduce the incidence of *Clostridium difficile*-associated diarrhoea in hospitalised patients (Plummer et al., 2004).

The specific objectives of this PhD study were to characterise a selection of these bacteria as follows:

Strain typing *Lactobacillus acidophilus*

- The major focus of the PhD study was *L. acidophilus* and evaluation of genetic typing methods for this species were investigated in order to:
  - Establish the sub-species level diversity of *L. acidophilus* using Randomly Amplified Polymorphic DNA (RAPD) fingerprint analysis
  - Develop robust molecular markers for detecting *L. acidophilus* at the species level
  - Test molecular markers in culture independent approach using a diverse range of samples to investigate *L. acidophilus* distribution

Minimum taxonomic criteria for bacterial genome sequence depositions

- During the course of the PhD, an announcement of the genome sequence of *L. acidophilus* strain 30SC was made (reference). When the 30SC genome was compared to the *L. acidophilus senso stricto* genomes as part of ongoing work, it became clear that the 30SC isolate was not a member of this species. The following analyses were performed to establish the correct species-identity of strain 30SC:
  - Analyse the phylogenetic placement of *L. acidophilus* 30SC using its recently published genome sequence
  - Construct *Lactobacillus* phylogenies using simple analyses based on the 16S rRNA gene sequence
  - Construct *Lactobacillus* phylogenies using simple analyses based on the sequence of other phylogenetic marker genes such as gyrase B
  - Set out a simple standard bioinformatic process that can be applied to newly generated genome sequences to ensure that they are accurately assigned to the genus or species level before deposition to a database and subsequent announcement

***Lactobacillus acidophilus* genomics and population structure**

- To enhance the strain level diversity analysis performed using RAPD, next-generation sequencing and cutting edge genomic comparative techniques were used to:
  - Obtain whole genome sequences for three isolates of *L. acidophius*; two commercial isolates (CUL21 and CUL60) and an isolate with an early deposition date (LMG 11428)
  - Use comparative genomics to establish the diversity of commercial isolates
  - Expand genomic information on *L. acidophilus* strains by resequencing the genomes of 28 *L. acidophilus* isolates
  - Apply Methyl Methane Sulphonate (MMS) to *L. acidophilus* to alkylate DNA and potentially induce mutations that could later be detected by DNA resequencing
  - Establish evolutionary history of *L. acidophilus* isolates
  - Investigate potential basis for limited isolate genetic diversity

Phenotypic diversity of *Lactobacillus*

- To complement the genetic analysis of *L. acidophilus,* phenotyping analysis was carried out to:
  - Examine species-level differences in *Lactobacillus* in the metabolism of carbohydrates using API 50CHL and evaluate as a method for identifying *L. acidophilus*
  - Evaluate the use of Matrix Assisted Laser Desorption/Ionisation – Time Of Flight (MALDI-TOF) mass spectrometry as a tool to examine the gross proteome of *Lactobacillus* isolates
  - Assess the use of MALDI-TOF MS as an identification tool for *Lactobacillus* isolates on both the species and strain levels
  - Establish the strain-level diversity of the gross proteome of *L. acidophilus*
  - Measure growth kinetics of *L. acidophilus* strains

Genomic diversity of bifidobacteria

- As a final component of this PhD study, preliminary genomic analysis of two *Bifidobacterium* isolates used by Cultech Ltd was carried out in order to:
  - Obtain whole genome sequences for two commercial bifidobacterial isolates, *Bifidobacterium bifidum* CUL20 and *Bifidobacterium animalis* subsp. *lactis* CU34
  - Use comparative genomics to establish the evolutionary history of commercial isolates
  - Develop robust molecular markers for detecting *Bifidobacterium bifidum and Bifidobacterium animalis* subsp. *lactis* at the species level
  - Test molecular markers in culture independent approach to establish efficacy and to investigate distribution of bifidobacteria.

2. ANALYSIS OF THE SUB-SPECIES LEVEL DIVERSITY OF *LACTOBACILLUS* STRAINS AND DEVELOPMENT OF NOVEL TOOLS FOR DETECTION OF *L. ACIDOPHILUS*

2.1. INTRODUCTION

Development and subsequent use of accurate tools for identifying lactobacilli at the species and sub-species level is vitally important, particularly of organisms that are used in commercial environments and food products such as starter-cultures for fermentation and probiotics. Sub-species level identification to identify strains of lactobacilli is important in the biotechnology industry, which requires tools to monitor, for example; the use of proprietary or patented strains, for quality control of batches of these organisms, or to distinguish between probiotic strains and those occurring naturally in the host gastrointestinal tract. It is also crucial to be able to compare clinical isolates and biotechnological strains and to be able to ascertain and monitor the genetic stability of commercial strains (Klein et al., 1998).

Molecular genotyping approaches are usually undertaken when studying the epidemiology of infectious disease pathogens; however, this study has used molecular genotyping to assess the intra-species diversity of a single probiotic bacterium, *L. acidophilus*, in the context of other closely-related species, belonging to the remarkably large and diverse *Lactobacillus* genus. Given that probiotic effect is often specific to a particular strain (Ouwehand et al., 2002, Luyer et al., 2005), accurate identification of cultures presumed to have probiotic properties is essential. It is accepted that probiotics should be classified and identified using internationally recognised methods such as Pulsed-Field Gel Electrophoresis, DNA-DNA hybridisation and 16S RNA gene sequencing (Pineiro and Stanton, 2007). Bacterial 16S rRNA gene sequencing is commonly used as a rapid and effective means of determining a genus- or species-level taxonomic identification although it provides little to no strain information (Naser et al., 2007). In biotechnological and industrial applications, identification is traditionally limited to these techniques and

conventional phenotypic identification using physiological parameters, rather than more modern methods practised in a research setting.

Traditionally, the identification of lactobacilli has been based mainly on fermentation of carbohydrates, morphology, and Gram staining, and these methods are still used. However, in recent years, the taxonomy has changed considerably with the increasing knowledge of the genomic structure and phylogenetic relationships between *Lactobacillus* species (Klein et al., 1998, Stiles and Holzapfel, 1997). Modern methods of strain typing are typically based on PCR, for example, random amplified polymorphic DNA (RAPD); or DNA fingerprinting method based on the restriction enzyme digestion of DNA, such as PFGE and ribotyping (Tynkkynen et al., 1999). In RAPD analysis, short DNA oligos of arbitrary sequence are used as PCR primers that produce amplification product patterns that are specific to each individual strain. PFGE relies on rare-cutting enzymes to digest genomic DNA into large fragments which are subsequently separated by electrophoresis, generating a fingerprint unique to each strain, while ribotyping uses rRNA genes or their spacer regions as probes that hybridize to genomic restriction fragments. Mainly due to methodological differences, discrepancies are often seen in the results of these techniques, and as such, any subspecies taxonomic assignation should be achieved as part of a polyphasic taxonomic approach (Vandamme et al., 1996).

Previous work has shown that when RAPD analysis was applied to a small collection of six reference isolates, *L. acidophilus* appeared to have very limited strain-level diversity (Mahenthiralingam et al., 2009). To increase the scope and depth of analysis beyond that of the previous study (Mahenthiralingam et al., 2009), this chapter presents a strain-level diversity analysis of a disparate group of LAB isolates, with particular focus on *L. acidophilus* reference and commercial isolates using RAPD analysis. An *L. acidophilus* specific PCR test was also developed in order to screen disparate niches for *L. acidophilus*, to expand and develop the range of isolates studied beyond those used in industrial

processes or collected as reference strains, and overall, to ensure that the range of isolates tested fully represent the strain level diversity of *L. acidophilus* as a LAB species.

2.1.1. SPECIFIC AIMS

The aims of this chapter were as follows:

- Investigate the sub-species level diversity of *L. acidophilus* using Randomly Amplified Polymorphic DNA (RAPD) fingerprint analysis.

- Develop robust molecular markers for detecting *L. acidophilus* at the species level.

- Develop molecular markers for each commercial *L. acidophilus* isolate using genome sequence information to investigate targets.

- Test molecular markers in culture independent approach using a diverse range of samples to investigate *L. acidophilus* distribution.

2.2. METHODS

2.2.1. STORAGE OF BACTERIAL ISOLATES

Stocks of bacterial isolates were prepared by resuspending fresh colonial growth from a pure culture plate into broth MRS (Oxoid, Basingstoke, UK) (de Man et al., 1960) containing 8% (v/v) DMSO or 15% (w/v) glycerol. Stocks were then maintained at – 80ºC.

2.2.2. GROWTH CONDITIONS

Unless otherwise stated, bacterial isolates were revived from frozen stocks and cultured on a MRS (de Man et al., 1960) agar at 37ºC. Liquid cultures (24 hours) were prepared by inoculating 3 ml of MRS broth media with a single colony from a revival plate, tubes were then incubated, statically at 37ºC for 24-48 hours, depending on the growth requirements of each isolate.

2.2.3. RAPID DNA EXTRACTION FROM COLONY MATERIAL

A single bacterial colony from a pure culture plate (with ≤ 72 hours' growth) was aseptically transferred to 50 µl of 5% (w/v) chelex® 100 resin solution (Biorad, Hertfordshire, UK, sterilised by autoclaving prior to use). DNA extraction was performed by heating the sample to 98°C on a heated block for a 5 minute cycle, then immediately placing the sample at 4ºC for 5 minutes. This process was repeated twice. Samples were then centrifuged briefly at 800 x *g* to sediment the resin and cellular debris and the supernatant containing the crude DNA removed for subsequent use.

2.2.4. GENERATION OF RAPD FINGERPRINTS

RAPD analysis was carried out as described by (Mahenthiralingam et al., 1996a). Primer 272, 5'- TGC GCG CGG G -3' (MWG Biotech, Covent Garden, London) was used for all reactions; reagents were from Qiagen (Qiagen, Crawley, Sussex, UK). Profile analysis was performed in 25 µl reaction mixtures containing: 1x PCR buffer, 1 x Q-solution, 3 mM

$MgCl_2$, 200 µm dNTPs mixture, 1.6 µM RAPD primer, 1 U of *Taq* polymerase and 2 µl of DNA template obtained by Chelex protocol. PCR thermal cycles were performed using a BioRad C1000 thermal cycler (BioRad, Hemel Hempstead, United Kingdom) as follows: 5 minutes at 94ºC, 4 cycles of 5 minutes at 36ºC, 5 minutes at 72ºC, 5 minutes at 94ºC, 30 cycles of 1 minute at 94ºC, 1 minute at 36ºC, 2 minutes at 72ºC followed by a final extension time of 10 minutes at 72ºC. 1 µl of the product was run on an Agilent Bioanalyser 2100 (Agilent, Santa Clara, US) using a DNA 7500 chip according to the manufacturer's protocol.

2.2.5. ANALYSIS OF RAPD FINGERPRINTS

Electropherogram data from the BioAnalyzer, in the form of a csv file, was converted to a GelCompar II (v6.6.8)-compatible format using a dedicated script provided by Applied Maths (Sint-Martens-Latem, Belgium) was applied to each csv-file to convert the profiles to a GelCompar-compatible format. Internal marker bands were then removed and band-searching set to a minimum profiling value of 2.0%. Similarity between fingerprints was calculated using the Pearson coefficient. Dendrograms were constructed by the unweighted pair group method with arithmetic means (UPGMA).

2.2.6. PCR AMPLIFICATION OF PAU REGIONS SPECIFIC TO *L. ACIDOPHILUS*

PAU gene amplification was used to identify the presence of *L. acidophilus.* PCR primers were generated using Primer3 (Rozen and Skaletsky, 2000) and are listed in Table 2.3. A 25 µl PCR mixture was set up as follows: 1x PCR buffer, 1x Q-solution, 100 µM final concentration of dNTPs, 0.4 µM final concentration of each primer, 2 µl of template DNA (extracted with Chelex (section 2.2.3), from total faecal DNA (Sections 2.2.10 and 2.2.11) or from the first round of PCR), and 1 U of *Taq* DNA polymerase. The nested PCR was carried out in two stages using a BioRad C1000 thermal cycler (BioRad, Hemel Hempstead, United Kingdom) the first using the program; 96°C for 1 min, then 30 cycles of 60 sec at 96ºC, 60 sec at 56ºC, 60 sec at 72ºC, then a final step at 72ºC for 10 min. The product from this PCR was then used as the template DNA for the second round of PCR using the program; 95°C for 2 min, then 35 cycles of 30 sec at 94ºC, 30 sec at 52ºC, 90 sec

at 72ºC, then a final step at 72ºC for 5 min. The resulting PCR products were visualised by agarose gel electrophoresis on a 1.5% (w/v) gel.

2.2.7. DETERMINATION OF THE PURE-CULTURE DETECTION LIMIT OF THE *L. ACIDOPHILUS* SPECIFIC MARKER PCR

A bacterial suspension of *L. acidophilus* NCFM was serially diluted in MRS broth. To enumerate, triplicate 10 µl drops were aspirated onto the surface of MRS agar plates and incubated at 37ºC for 24 hours. Individual colonies were counted and the number of viable cells calculated and expressed as colony forming units per ml (CFU/ml). DNA was extracted from suspensions at each dilution using the following method: Cells were pelleted by centrifugation 1,200 x g for 10 min. The pellet was resuspended in 100 µl of TE (10 mM Tris-Cl pH8, 10 mM EDTA, pH 8) and transferred to a 2 ml screw-cap microcentrifuge tube containing approximately 0.5 ml of 0.1 mm diameter Zirconium beads (Biospec Products, Bartlesville, Oklahoma) and 500 µl lysis buffer (50 mM Tris-Cl pH8, 70 mM EDTA pH8, 1% (w/v) SDS) with 0.5 mg/ml Pronase (Roche, Hertfordshire, UK). The bacteria were then lysed by a 10 sec pulse on a mini bead-beater device (Biospec Products) and incubation at 37˚C for 1 hour to digest proteins. 200 µl of saturated ammonium acetate was then added, followed by a 5 sec pulse on the bead-beater device. The tubes were then centrifuged for 1 min at 16,100 x g, followed by the addition of 600 µl of chloroform. The tubes were pulsed on the bead-beater device for 5 sec and then phases were separated by centrifugation for 7 min at 16,100 x g. Genomic DNA was collected from the cleared aqueous phase by ethanol precipitation. After the DNA pellet was washed with 70% (v/v) ethanol and vacuum dried it was resuspended in 50 – 300 µl low EDTA-TE (10 mM Tris-Cl pH 8, 0.1 mM EDTA) with RNase A at 0.5 µg/ml. The extracted DNA was then used as template DNA as described in (Section 2.2.6).

2.2.8. ISOLATING LAB FROM THE FAECES OF WILD RODENTS

Wild rodents were trapped near Llysdinam in Wales, UK using baited live-capture Longworth traps that were set overnight. Faeces was collected from the traps aseptically and stored in 1.5ml Eppendorf tubes at -20°C. LAB were isolated from mouse faeces by

disrupting a thawed faecal pellet in 1.5ml Maximum Recovery Diluent (MRD; Oxoid, Basingstoke, UK). Serial dilutions were then plated onto MRS agar supplemented with 120 units per ml of Polymixin B (Sigma Aldrich, Gillingham, UK), and incubated at 37°C for 72 hours. Fresh colonies were simultaneously picked into 50 µl of 5% (w/v) Chelex resin for RAPD typing and testing with PAU-based specific marker PCR and into MRS broth for growth before storage at -80°C (Section 2.2.1).

2.2.9. BACTERIAL 16S RRNA GENE AMPLIFICATION OF LAB ISOLATES CULTIVATED FROM WILD RODENTS

16S rRNA gene amplification and sequencing was used to identify the bacteria isolated from wild rodent faeces. Primers 27F (5'- AGA GTT TGA TC(AC) TGG CTC AG -3') and 1492R (5'- GGT TAC CTT GTT ACG ACT T -3') (Lane, 1991) were used to amplify the near full length 16S rRNA gene as follows. A 50 µl PCR mixture was set up as follows: 1X PCR buffer, 1 X Q-solution, 100 µM final concentration of dNTPs, 0.4 µM final concentration of each primer, 3 µl of Chelex template DNA, and 1 U of *Taq* DNA polymerase. PCR was carried out in BioRad C1000 thermal cycler (BioRad, Hemel Hempstead, United Kingdom) as follows: 95°C for 2 min, then 35 cycles of 30 sec at 94ºC, 30 sec at 52ºC, 90 sec at 72ºC, then a final step at 72ºC for 5 min. The resulting PCR product was analysed by agarose gel electrophoresis and sequenced on the forward strand using primer 27F and the reverse strand with primer 1492R in order to obtain a full length sequence of the 16S rRNA gene. Sequencing reactions were carried out using Applied Biosystems Big Dye Terminator ready reaction mix version 3.1, with subsequent analysis on an Applied Biosystems ABI-Prism 3100 automated sequencer. Alignments were constructed with BioEdit v7.2.1(Ibis Biosciences, Carlsbad, US) and phylogenetic trees were constructed using MEGA 4 (Tamura et al., 2007).

2.2.10. EXTRACTION OF TOTAL FAECAL DNA FROM RODENT FAECAL PELLETS

Total faecal DNA was extracted from the faecal of wild rodents and rodents on the Lab4® probiotic feeding trial using a FastDNA Spin Kit for Soil (Qbiogene Inc., Carlsbad, CA) and following the manufacturer's instructions.

2.2.11. EXTRACTION OF TOTAL FAECAL DNA FROM HUMAN FAECES

DNA was extracted from human faecal material using the QIAamp® DNA Stool Mini Kit (Qiagen, Crawley, Sussex, UK) following the manufacturer's instructions, modified to include an additional disruption step. 0.5 g of 0.1 mm zirconium beads (Biospec Products, Bartlesville, Oklahoma) were added to each sample, which was subsequently disrupted in three one-minute bursts using a Fastprep® machine with a 24x2ml adaptor (MP Biomedicals, Solon, US).

2.3. RESULTS

For clarity, the results were divided into three parts, the first (Section 2.3.1) concerns the application of RAPD profile similarity analysis to a collection of LAB isolates, with particular emphasis on *L. acidophilus*, which included two *L. acidophilus* isolates from the Cultech Lab4® probiotic supplement (CUL21 and CUL60). The assembled collection of *L. acidophilus* isolates (Table 2.1) were originally isolated from a relatively limited range of locations, with the majority from commercial products (dairy, probiotic and other fermented products) and Cultech products, and were all associated with human processes. The second part (Section 2.3.2) describes the development of a reliable method for detecting *L. acidophilus* from collection both cultured and uncultured samples. The third (Section 2.3.2) seeks to expand the scope of the isolate collection by applying the developed method to samples from a variety of hosts, also allowing investigation into the distribution of *L. acidophilus* in various hosts.

2.3.1. RAPD FINGERPRINTING OF LAB ISOLATES

2.3.1.1. Validation of RAPD fingerprinting method for LAB isolates

A broad collection of 72 LAB isolates from a variety of isolation sources (Table 2.1) was used to assess the discriminatory ability and reproducibility of the RAPD fingerprinting method when applied to a large collection of LAB isolates, with the broad emphasis on assessing the genetic diversity of LAB isolates and primary goal of defining overall diversity within *L. acidophilus*. The use of primer 272 (Mahenthiralingam et al., 1996b) for RAPD fingerprinting of LAB was validated by generating least five PCR products of differing size for each of the isolates. The use of the Bioanalyzer greatly reduced between-gel profile variation seen between standard 1.5% (w/v) agarose gels, indicated by repeated typing of a control strain showing at least 90.6% profile stability (Figure 2.1). The stability and reproducibility of the control fingerprint over time shows the value of RAPD as strain typing method for analysing isolates over a long time period.

Table 2.1: LAB isolates, their RAPD profile types and isolation sources

Species (16S rRNA gene sequence)	Species based on RAPD profile	Isolate name	Source or product from which the isolate was cultivated	RAPD profile type
L. acidophilus	-	BFT2	Commercial probiotic	1
L. acidophilus	-	C21	Cultech Ltd.	1
L. acidophilus	-	C46	Cultech Ltd	1
L. acidophilus	-	C47	Cultech Ltd.	1
L. acidophilus	-	C49	Cultech Ltd.	1
L. acidophilus	-	C77	Cultech Ltd.	1
L. acidophilus	-	C85	Cultech Ltd.	1
L. acidophilus	-	CUL21	Cultech Ltd.	1
L. acidophilus	-	CUL60	Cultech Ltd.	1
L. acidophilus	-	Rm344	Cultech Ltd.	1
L. acidophilus	-	Rm345	Cultech Ltd.	1
L. acidophilus	-	CulT2	Cultech Ltd.	1
L. acidophilus	-	HBAP T1	Commercial probiotic	1
L. acidophilus	-	HBCA	Commercial probiotic	1
L. acidophilus	-	LAB 283	Peter Vandamme (Unpublished)	1
L. acidophilus	-	LAB 444	Peter Vandamme (Unpublished)	1
L. acidophilus	-	LAB 64	Peter Vandamme (Unpublished)	1
L. acidophilus	-	LAB 66	Peter Vandamme (Unpublished)	1
L. acidophilus	-	LAB 67	Peter Vandamme (Unpublished)	1
L. acidophilus	-	LAB 683	Peter Vandamme (Unpublished)	1
L. acidophilus	-	LAB 69	Peter Vandamme (Unpublished)	1
L. acidophilus	-	LAB 76	Peter Vandamme (Unpublished)	1
L. acidophilus	-	LAB 77	Peter Vandamme (Unpublished)	1
L. acidophilus	-	LAB 79	Peter Vandamme (Unpublished)	1
L. acidophilus	-	LAB 74	Peter Vandamme (Unpublished)	1
L. acidophilus	-	NCFM	Reference (Human)	1
L. acidophilus	-	LMG 11428	Reference (Rat faeces)	1
L. acidophilus	-	LMG 11430	Reference (Human)	1
L. acidophilus	-	LMG 11466	Reference	1
L. acidophilus	-	LMG 11467	Reference (Human)	1
L. acidophilus	-	LMG 11469	Reference (Rat intestine)	1
L. acidophilus	-	LMG 11470	Reference	1
L. acidophilus	-	LMG 11472	Reference	1
L. acidophilus	-	LMG 13550	Reference (Human)	1
L. acidophilus	-	LMG 9433	Reference (Human)	1
L. acidophilus	-	TT1	Commercial probiotic	1
L. brevis	-	LMG 6906	Reference (Human faeces)	2
L. casei	-	P7T1	Commercial	3

Table 2.1: LAB isolates, their RAPD profile types and isolation sources (cont.)

Species (16S rRNA gene sequence)	Species based on RAPD profile	Isolate name	Source or product from which the isolate was cultivated	RAPD profile type
L. casei	-	C48	Cultech Ltd.	4
L. casei	-	C65	Cultech Ltd.	5
L. casei	-	LMG 6904	Reference (Cheese)	6
L. acidophilus	*L. casei*	TBCC	Commercial probiotic	6
L. gasseri	-	C44	Cultech Ltd.	7
L. gasseri	-	C63	Cultech Ltd.	7
L. gasseri	-	C67	Cultech Ltd.	7
L. gasseri	-	C64	Cultech Ltd.	8
L. gasseri	-	LMG 9203	Reference (Human)	9
L. gasseri	-	SSMB	Commercial probiotic	10
L. jensenii	-	C66	Cultech Ltd.	11
L. acidophilus	*L. jensenii*	C68	Cultech Ltd.	12
L. jensenii	-	C72	Cultech Ltd.	12
L. johnsonii	-	LMG 9436	Reference (Human blood)	13
L. paracasei subsp. *paracasei*	-	LMG 7955	Reference	14
L. plantarum	-	C13	Cultech Ltd.	15
L. plantarum	-	LMG 6907	Reference (Pickled cabbage)	15
L. plantarum	-	HBRAT1	Commercial	16
L. rhamnosus	-	FMDT2	Commercial probiotic	17
L. rhamnosus	-	GG	Commercial probiotic	18
L. rhamnosus	-	QAPT1	Commercial probiotic	19
L. salivarius	-	CulT1	Cultech Ltd.	20
L. suntoryeus	-	C78	Cultech Ltd.	21
L. suntoryeus	-	C80	Cultech Ltd.	21
L. suntoryeus	-	C81	Cultech Ltd.	21
L. suntoryeus	-	C82	Cultech Ltd.	21
L. suntoryeus	-	C84	Cultech Ltd.	21
L. suntoryeus	-	FMDT1	Commercial probiotic	21
L. suntoryeus	-	HS1	Cultech Ltd.	21
L. suntoryeus	-	HS3	Cultech Ltd.	21
unknown	*L. suntoryeus*	HT2	Commercial probiotic	21
L. suntoryeus	-	HBRAT2	Commercial probiotic	22
L. suntoryeus	-	P7T2	Commercial probiotic	22
L. suntoryeus	-	QAPT2	Commercial probiotic	22
Pediococcus pentosaceus	-	HBRAT3	Commercial probiotic	23
Enterococcus faecalis	-	C22	Cultech Ltd.	24
Enterococcus faecium	-	LMG 14205	Reference	25

2.3.1.2. Application of RAPD fingerprint analysis to LAB isolates

Table 2.1 details the RAPD type assigned to each isolate using RAPD primer 272. RAPD profile types were differentiated on the basis of 75% fingerprint similarity. This level of similarity was chosen as a cut-off value for strain type designation based on the findings from RAPD analysis surveys of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients (Campbell et al., 2000). It was also supported by empirical evidence, with the value of 75% correlating to the assessment of profile types that were visually distinct (Figure 2.2).

Three RAPD profile types, 1, 6, and 12 (Table 2.1), were initially associated with isolates identified by partial 16S rRNA gene sequence analysis as *L. acidophilus*. The majority of *L. acidophilus* isolates generated a single RAPD profile (type 1) with profile types 6 and 12, in each case represented by just a single isolate. *L. acidophilus* TBCC (isolated from a commercial probiotic product) generated a RAPD profile type that was subsequently found to be shared by *L. casei* LMG 6904 (Table 2.1). *L. acidophilus* C68 (isolated by Cultech Ltd) shared a profile type with *L. jensenii* C72. Accordingly, species designations were updated for these isolates, and further proof of the correct reclassification of *L. acidophilus* TBCC to *L. casei* TBCC was also observed after carbohydrate fermentation profiling (see Chapter 5; Figure 5.2).

All isolates of *L. acidophilus* that were unequivocally confirmed as this species type were found to have the same RAPD profile type (type 1), including the two commercial strains from the Cultech Lab4® probiotic supplement, *L. acidophilus* CUL21 and *L. acidophilus* CUL60. In comparison, other LAB tested showed a variety of different RAPD profile types per species (Table 2.2). *L. casei* and *L. gasseri* displayed the greatest within-species RAPD profile variability, with the majority of isolates tested generating a distinct RAPD profile (Table 2.2). However, *L. suntoryeus* showed only two RAPD profile types over 12 isolates tested (Table 2.2).

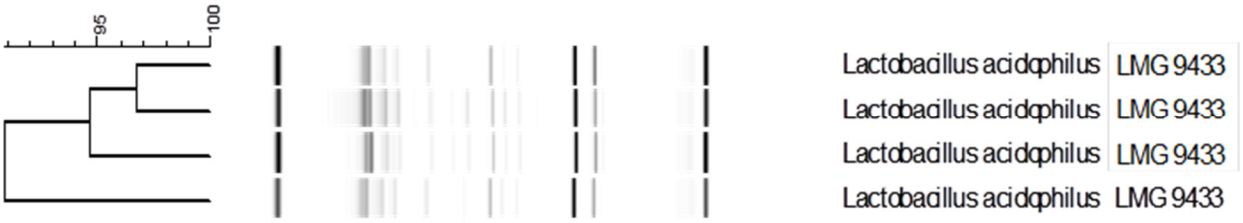


Figure 2.1: Clustered RAPD profiles from multiple re-typing of *L. acidophilus* LMG 9433^T as an internal control.

Re-typed *L. acidophilus* LMG 9433^T isolates cluster together at greater than 90.6%. Clustering analysis was performed using GelCompar II (Applied Maths, Belgium). Pearson correlation similarity coefficient with a UPGMA dendrogram type was used, and position tolerance optimisation was set to 0.5%.

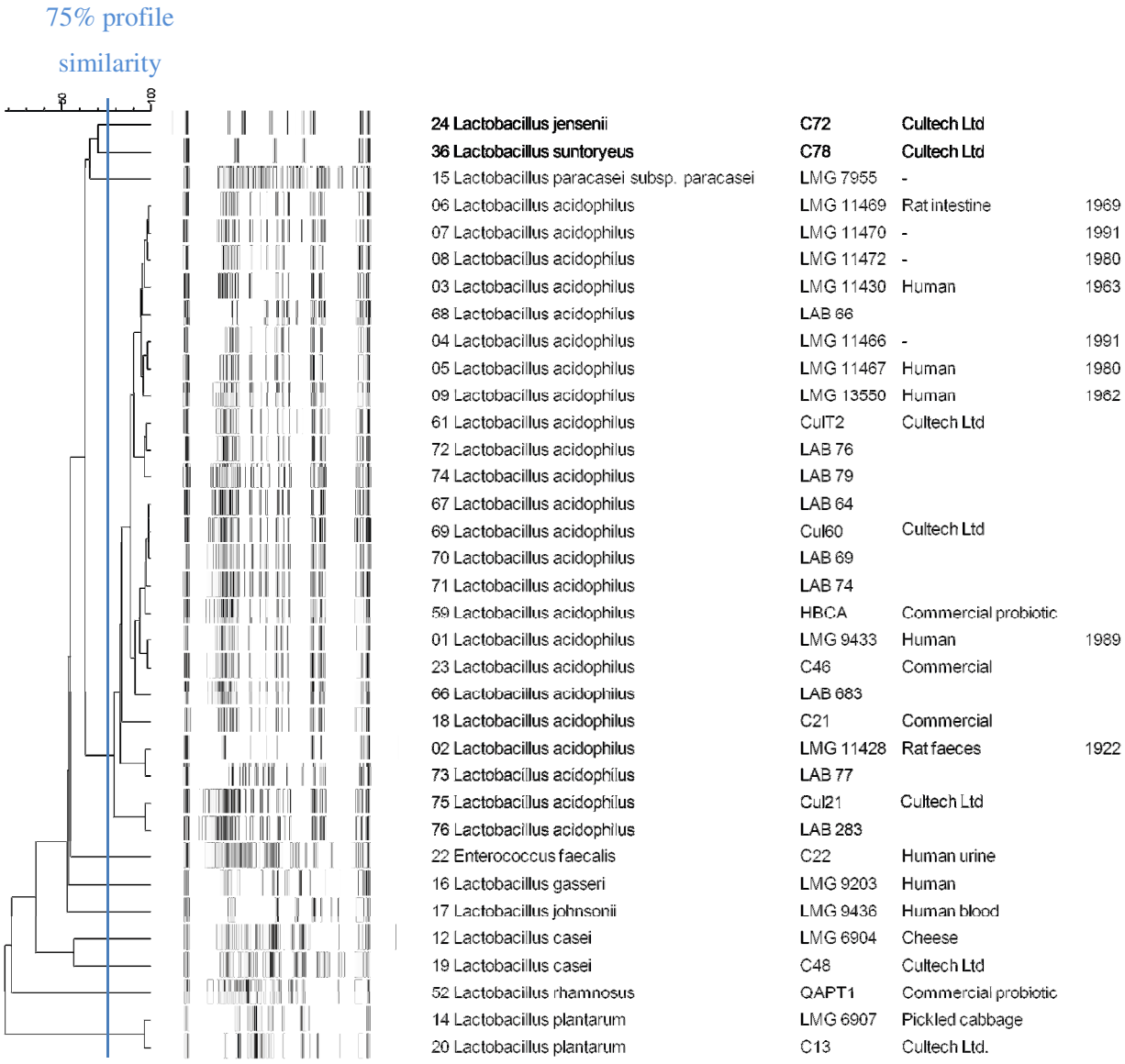


Figure 2.2: Clustered RAPD profiles of *Lactobacillus* and *Enterococcus* isolates from a range of isolation dates and sources.

L. acidophilus isolates clustered at greater than 79%. Cluster analysis was performed using GelComparII (Applied Maths, Belgium). Pearson correlation similarity coefficient with a UPGMA dendrogram type was used, with position tolerance optimisation set to 0.5%. Strain-type profile similarity cut-off (75%; Section 2.3.1) is indicated.

Table 2.2: RAPD profile type represented by LAB

Species	Isolates	RAPD profile types
L. acidophilus sensu stricto	33	1
L. brevis	1	1
L. casei	5	4
L. gasseri	6	4
L. jensenii	2	1
L. johnsonii	1	1
L. paracasei subsp. *paracasei*	1	1
L. plantarum	3	2
L. rhamnosus	3	3
L. salivarius	1	1
L. suntoryeus	12	2
P. pentosaceus	1	1
E. faecalis	1	1
E. faecium	1	1

2.3.2. DEVELOPMENT OF A SPECIFIC TEST FOR *L. ACIDOPHILUS*

2.3.2.1. Development of the Potentially Autonomous Units (PAUs) of *L. acidophilus* as species specific markers

The three PAU regions of *L. acidophilus* were selected as prospective targets for development as species-specific markers because of their unique nature within the *L. acidophilus* NCFM genome (Figure 2.3). As an initial screening procedure, the DNA sequences corresponding to target ORFs within the PAU region (Table 2.3) were searched against the non-redundant nucleotide database at the National Centre for Biotechnology Information (NCBI) to establish their specificity. Only sequences belonging to *L. acidophilus* were returned as similar (100% coverage, 100% identity, e=0), indicating that the only record of the PAU regions in the NCBI's nucleotide database were from *L. acidophilus*.

The LBA0479 open reading frame within the Pau3 region was selected as a gene target and a simple PCR test for PAU DNA was devised using primers FPau3 and RPau3 (Table 2.3). Initial testing indicated that these PCR primers would allow detection of approximately 5000 colony forming units (cfu) in pure culture. In order to decrease the number of *L. acidophilus* cfu able to be detected by this test, a further, nested, PCR primer set was designed. The target sequences of these PCR primers are interior to FPau3 and RPau3, and coupled with a second thermal cycle, decreased the detection limit of the PAU specific marker test to 50 cfu in pure culture (Figure 2.4) .

The specificity of the PAU specific marker system was tested using DNA extracted from relevant LAB cultures. The test panel was composed of DNA extracted from a panel of 32 LAB species and a single isolate of *B. cepacia* J2315 (Table 2.4). The panel of 32 LAB comprised of 25 *L. acidophilus* isolates from various isolation sources and dates, as well as reference isolates of *L. casei*, *L. plantarum*, *L. paracasei* subsp. *paracasei*, *L. gasseri* and

L. johnsonii (Table 2.1). Also included were two *L. acidophilus* isolates reclassified by RAPD profile similarity analysis; *L. casei* (*L. acidophilus*) TBCC and *L. jensenii* (*L. acidophilus*) C68. For the purpose of testing the sensitivity and specificity of the *L. acidophilus* specific marker system, the 25 isolates that generated a type 1 RAPD profile were considered to be *L. acidophilus sensu stricto*

The PAU-based *L. acidophilus* specific marker test was 100% specific for identification of *L. acidophilus* isolates when compared with other LAB species. All reference isolates from other LAB species failed to generate a positive PCR product, as did *B. cepacia*. Also, the two re-classified isolates; *L. casei* (*L. acidophilus*) TBCC and *L. jensenii* (*L. acidophilus*) C68, did not amplify the specific marker region. The PAU3 PCR was not successful at detecting all *L. acidophilus* isolates of RAPD profile type 1 however, producing a sensitivity of 92% within this species, with isolates LMG 11466 and LMG 11469 proving negative for amplification of the specific LBA0479 ORF (Table 2.4). After resequencing the genomes of these isolates, they were found to lack the PAU3 region (See Section 4.3.2.2.2). All commercial commercial isolates, including *L. acidophilus* CUL21 and CUL60 from the Lab4® complex probiotic, were found to amplify the specific marker region.

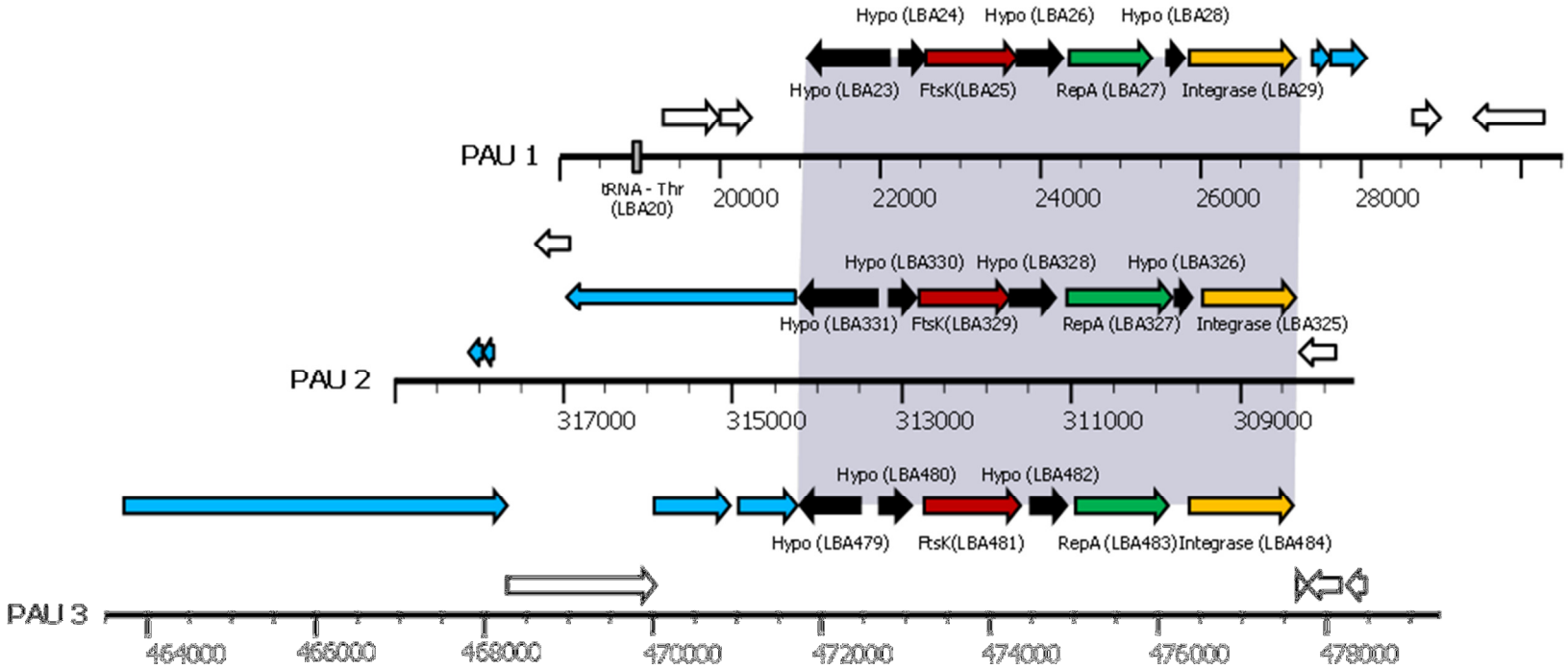


Figure 2.3: Three PAU regions of the *L. acidophilus* NCFM genome

The genome is represented by a black line, and base-pair intervals are shown in bp. Three PAUs were aligned at the 5′ end of the integrase, which is shown as an orange arrow, and are drawn to scale. RepA is represented by green arrows and FtsK is represented by red arrows. ORFs with no predicted functions that are assumed to be part of a PAU are shown as black arrows. Blue ORFs indicate proteins potentially involved in unit stabilization. Adjacent ORFs are shown as white arrows. tRNAs are drawn as gray boxes on the genome line.

Table 2.3: PCR primer sequences for *L. acidophilus* specific PCR, based on PAU regions

Primer Name	ORF[a]	ORF Function	ORF Size (bp)	Orientation	Tm	Sequence	Product size (bp)
FPau3	LBA479	hypothetical	702	Forward	58.75	TGATAATGACCCAATAACAATCG	535
RPau3				Reverse	60.73	GGTCAAGACTGTGTGTAACAATGG	
FPau3_NESTED	LBA479	hypothetical	702	Forward	53.14	TCCTAGAATGGTAAGAGATTGGCGGGA	405
RPau3_NESTED				Reverse	53.82	AGCAAATGCTGTGAAGCATCAGGTGT	

[a]ORF designations extracted from *L. acidophilus* NCFM genome sequence (GenBank accession: CP000033)

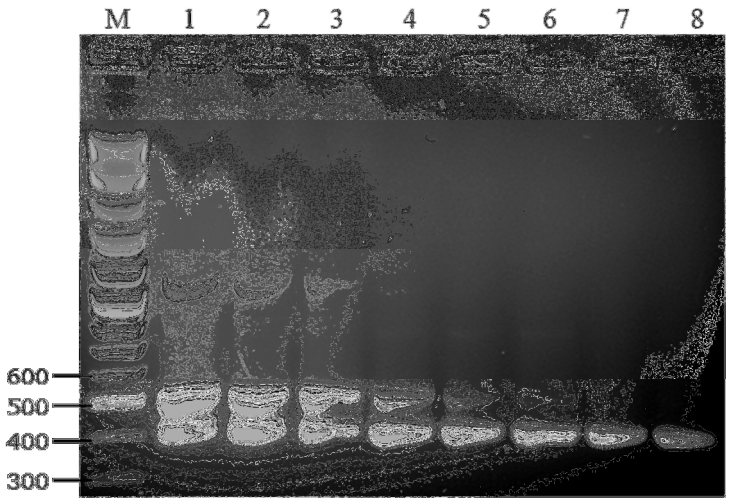


Figure 2.4: Detection limits of *L. acidophilus* specific markers in pure culture.

Number of input colony forming units (cfu) into DNA extraction before PCR decreases 10-fold moving from left-most lane to right-most lane. Lane 1= 5 x 10^8 cfu, lane 2 = 5 x 10^7 cfu, lane 3 = 5 x 10^6 cfu, lane 4 = 5 x 10^5 cfu, lane 5 =5 x 10^4 cfu, lane 6 = 5 x 10^3 cfu, lane 7 = 500 cfu, lane 8 = 50 cfu. Marker lane is denoted M and relevant molecular weight marker sizes are included in bp.

Table 2.4: Specificity of PAU-based *L. acidophilus* specific markers

Species	Isolate	PAU3 PCR
L. acidophilus	LMG 9433	+
L. acidophilus	LMG 11428	+
L. acidophilus	LMG 11430	+
L. acidophilus	LMG 11470	+
L. acidophilus	LMG 11472	+
L. acidophilus	LMG 11466	-
L. acidophilus	LMG 11469	-
L. acidophilus	LMG 13550	+
L. acidophilus	CUL60	+
L. acidophilus	CUL21	+
L. acidophilus	C21	+
L. acidophilus	C46	+
L. acidophilus	C47	+
L. acidophilus	C49	+
L. acidophilus	LAB 66	+
L. acidophilus	LAB 69	+
L. acidophilus	LAB 76	+
L. acidophilus	LAB 79	+
L. acidophilus	LAB 444	+
L. acidophilus	LAB 283	+
L. acidophilus	NCFM	+
L. acidophilus	Rm344	+
L. acidophilus	Rm345	+
L. acidophilus	CulT2	+
L. acidophilus	HBCA	+
L. casei (*L. acidophilus*)[a]	TBCC	-
L. casei	LMG 6904	-
L. plantarum	LMG 6907	-
L. paracasei subsp. *paracasei*	LMG 7955	-
L. jensenii (*L. acidophilus*)[a]	C68	-
L. gasseri	LMG 9203	-
L. johnsonii	LMG 9436	-
Burkholderia cepacia	J2315	-

[a] Species names given in brackets were assigned before RAPD profile similarity analysis.

2.3.3. PAU-BASED SPECIFIC MARKERS IN CULTURE-DEPENDENT AND CULTURE-INDEPENDENT APPLICATIONS

2.3.3.1. Isolation of *L. acidophilus* from mouse faeces

With the intention of expanding the host diversity of the LAB examined in this study, the PAU-based specific markers were applied in a culture-dependent approach to LAB isolated from faecal pellets of wild rodents. A total of 45 wild rodents were trapped over a 24 hour period consisting of; 25 bank voles (*Myodes glareolus*), 11 wood mice (*Apodemus sylvaticus*), six yellow-necked mice (*Apodemus flavicollis*), and three common shrews (*Sorex araneus*). Faecal pellets from traps containing more than one rodent were discarded. Faecal pellets were obtained from 37 of the traps. Briefly, the faecal pellets collected from wild rodents were homogenised and diluted before spreading onto MRS agar to enrich for growth of LAB. Colonies were screened based on their morphology, and any resembling *L. acidophilus* were selected for further analysis. In addition, colonies were also picked at random to populate the LAB isolate collection with a diversity of isolates. In total, 70 colonies were isolated from 37 wild rodent faecal pellets.

Immediately prior to storage of the picked colonies, a rapid DNA extraction using Chelex® resin was performed and this DNA was used as input for PCRs amplifying part of the 16S rRNA gene and the *L. acidophilus* specific PAU marker region. Additionally, a RAPD-PCR using PCR primer 272 was undertaken on each colony. Total faecal DNA was also extracted from any remaining faecal material and was used as input for a nested PCR targeting the *L. acidophilus* specific PAU marker region to detect the presence of *L. acidophilus* using a culture-independent approach.

A phylogeny based on the partial 16S rRNA gene sequences of the isolated LAB showed the dominant isolated species to be *L. animalis* and *L. murinus*. Isolates belonging to the *L. acidophilus* group were captured, but these belonged to *L. intestinalis* rather than *L. acidophilus* species (Figure 2.5). No *L. acidophilus* was isolated, with, no isolates testing

positive for the PAU-based *L. acidophilus* marker region and no RAPD profile matching the unique *L. acidophilus* profile type (type 1, Table 2.1, Figure 2.1). Finally, when applied to total faecal DNA extracted from wild rodent faeces in a culture-independent approach, the PAU-based *L. acidophilus* specific marker PCR detected no *L. acidophilus* at either the first round (detects more than approximately 5,000 cfu) or the second round (detects more than approximately 50 cfu).


4104
3502
4103
3501
Lactobacillus intestinalis (AJ306299)
0202
Lactobacillus amylovorus (AY944408)
Lactobacillus crispatus (AF257097)
Lactobacillus acidophilus (AY773947)
Lactobacillus helveticus (AM113779)
1101
1901
Lactobacillus johnsonii (AJ002515)
Lactobacillus gasseri (AF519171)
3701
0503
3803
Lactobacillus delbrueckii (AY050173)
Lactobacillus reuteri (L23507)
1001
2402
2401
4904a
4906b
Lactobacillus rhamnosus (D16552)
Lactobacillus paracasei (AB181950)
Lactobacillus casei (AF469172)
Lactobacillus brevis (M58810)
Lactobacillus plantarum (AJ965482)
Lactobacillus salivarius (AF089108)
0506
4901
Lactobacillus murinus (AJ621554)
7301
4905
Lactobacillus animalis (AB326350)
3401
7601
3001
3201
7201
6201
0401
Enterococcus faecium (AJ301830)
2718
7502
2703
6402
6407
2702
Enterococcus faecalis (AB012212)
2701
6408
6406
2704
Staphylococcus warneri (L37603)
6405
6404
Pseudomonas aeruginosa (AB037555)
0.05
L. acidophilus group
L. casei group
L. salivarius group

← Previous page

Figure 2.5: LAB isolated from wild rodent faeces

Neighbour-joining tree based on the 16S rRNA gene sequences of LAB isolated from wild rodents. Isolates (coded in black text) are compared to relevant reference sequences (red text, GenBank accession nos. indicated). Above-species-level groups are indicated with a brace. Alignments over 398 positions, 1000 replicates generated bootstrap figures, scale is indicated in substitutions-per-site. The tree is rooted with *Pseudomonas aeruginosa* 16S rRNA gene sequence.

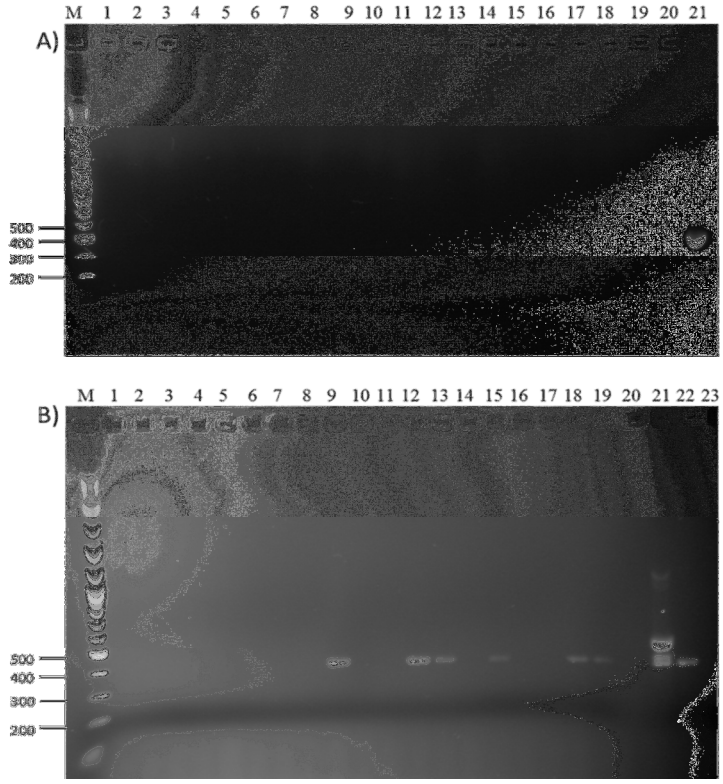
2.3.3.2. Culture-independent application of *L. acidophilus* specific PAU-based markers

To further test the ability of the PAU-based marker test to detect *L. acidophilus* DNA in total faecal DNA from a range of host species and systems, total faecal DNA was prepared from (i) adults from the Republic of Côte d'Ivoire, (ii) healthy human males enrolled on a probiotic (Lab4®) feeding trial, (iii) rats enrolled on a Lab4® probiotic feeding trial, and (iv) wild rodent faeces, and used a template DNA for the *L. acidophilus* specific PAU-based marker PCR.

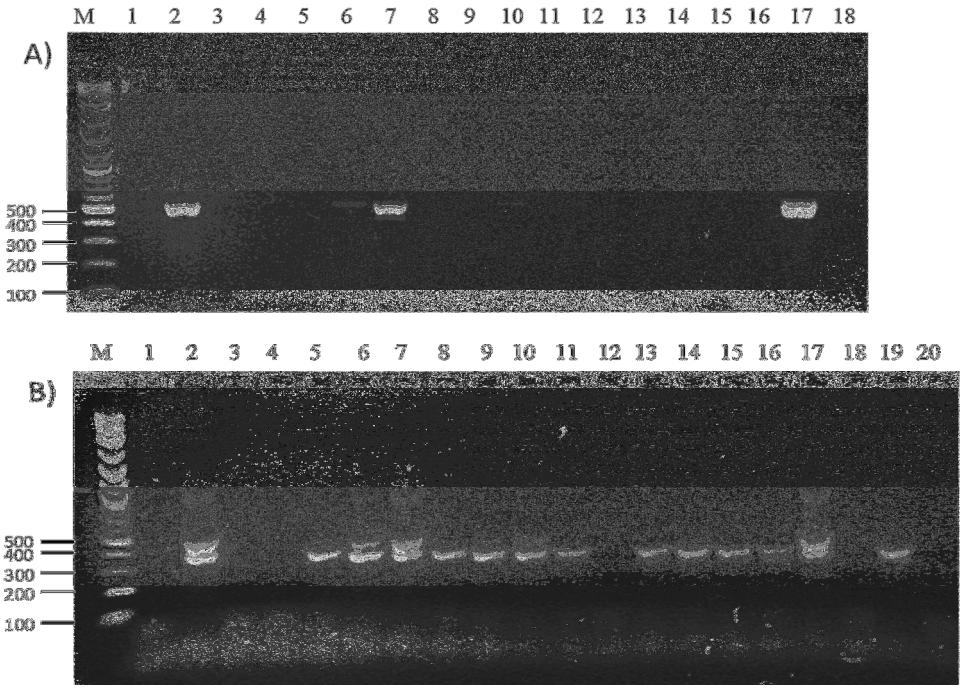
No adults from Republic of Côte d'Ivoire tested positive for more than 5,000 cfu of *L. acidophilus* (Figure 2.6, panels (i)A and (i)B). After the second round of PCR, 6/19 individuals tested positive for PAU DNA indicating that between 50-5,000 cfu of *L. acidophilus* was present in these samples (Figure 2.6, panels (i)B, lanes 9, 12, 13, 15, 18 and 19). When the same test was applied to sixteen healthy human adults enrolled on a Lab4® probiotic feeding trial (pre-feeding samples), four participants tested positive for PAU DNA representative of more than 5,000 cfu of *L. acidophilus* being present (Figure 2.6, panel (ii)A, lanes 2, 6, 7 and 10). An additional eight tested positive for PAU DNA when the second round PCR was carried out to detect between 50-5,000 cfu of *L. acidophilus* (Figure 2.6, panel (ii)B, lanes 5, 8, 9, 11, 13, 14, 15 and 16).

When applied to total faecal DNAs from rats that were being fed the probiotic Lab4® mixture, no individuals were found to be carrying PAU DNA representative of greater than 5,000 cfu of *L. acidophilus* (Figure 2.7, panel A). However, the majority of rats (10/11) were found to be carrying the PAU marker at a level representing between 50 and 5,000 cfu of *L. acidophilus* (Figure 2.7, panel B). When the same test was applied to total faecal DNA extracted from wild rodents, no detectable levels of *L. acidophilus* were seen with the specific marker test.

(i)


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
A)
500
400
300
200
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
B)
500
400
300
200

(ii)


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
A)
500
400
300
200
100
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
B)
500
400
300
200
100

← Previous Page

Figure 2.6: Examining adult total faecal DNA from (i) Adults from Republic of Côte d'Ivoire and (ii) healthy human males enrolled on a probiotic (Lab4®) feeding trial (pre-feeding), for the presence of *L. acidophilus* using PAU-based *L. acidophilus* specific marker

Panel (iA) shows PCR products from 19 total faecal DNAs (lanes 1-19), a negative PCR water control (lane 20) and *L. acidophilus* NCFM DNA as a positive control (lane 21). Panel (iB) shows the same, with an additional *L. acidophilus* NCFM DNA positive control for the second round of PCR (lane 22) and an additional water PCR control for the second round of PCR (lane 23). Panel (iiA) shows 16 total faecal DNAs (lanes 1-16), an *L. acidophilus* NCFM DNA as a positive control (lane 17) and a negative PCR water control (lane 18). Panel (iB) shows the same, with an additional *L. acidophilus* NCFM DNA positive control for the second round of PCR (lane 19) and an additional water PCR control for the second round of PCR (lane 20). Lane M contains molecular size marker with sizes of relevant bands given in bp.

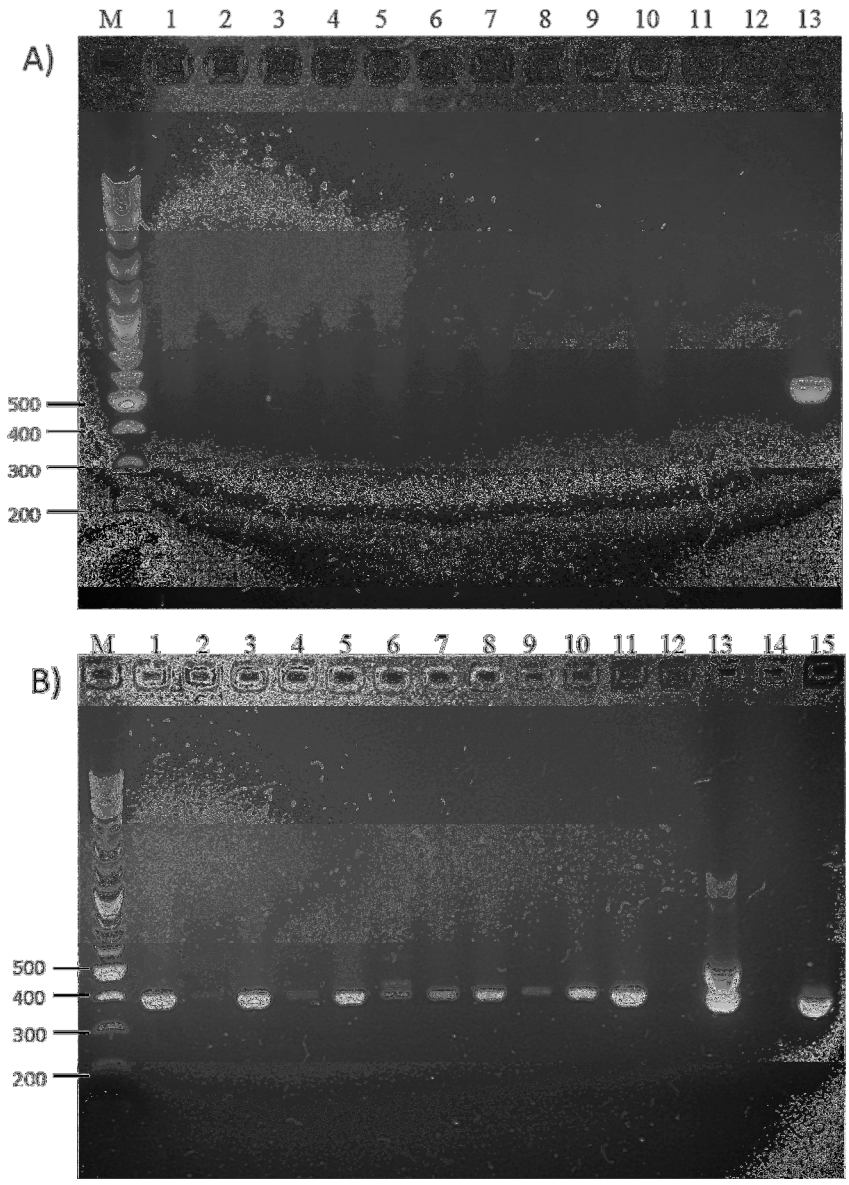


Figure 2.7: Examining total faecal DNA from rats after feeding of the Lab4® probiotic (Lab4®) for the presence of *L. acidophilus* using PAU-based *L. acidophilus* specific marker

Panel A shows PCR products from 11 total faecal DNAs (lanes 1-11), a negative PCR water control (lane 12) and *L. acidophilus* NCFM DNA as a positive control (lane 13). Panel B shows the same, with an additional *L. acidophilus* NCFM DNA positive control for the second round of PCR (lane 15) and an additional water PCR control for the second round of PCR (lane 14)

2.4. DISCUSSION

2.4.1. STRAIN TYPING LAB USING RAPD PROFILE SIMILARITY ANALYSIS

2.4.1.1. Typing *Lactobacillus* isolates

PCR-electrophoresis-based strain typing methods, particularly RAPD, have previously been criticised for their low repeat reproducibility (Maukonen et al., 2003). The reproducibility of RAPD profiles for the same isolate, generated at multiple time points and on different microfluidics chips, showed that the use of the Bioanalyzer microfluidics platform in this study reduced repeat variability to less than 10%, less than half the profile variability expected to be seen within a single profile type (Figure 2.1). This allowed RAPD to be performed on a large collection of LAB isolates by enabling accurate comparisons of RAPD profiles generated on different microfluidics chips. This supported previous work that showed the microfluidics system was advantageous over agarose gel electrophoresis (Cooper et al., 2008), where comparisons can only be drawn with any accuracy from a small dataset on a single agarose gel.

RAPD profile similarity analysis was successfully applied to 75 LAB isolates, belonging to 14 distinct species. High quality RAPD profiles for all 75 LAB isolates, which were originally isolated from a wide range of sources and years, were generated and subsequently compared to examine the genetic diversity of LAB isolates, with particular emphasis on *L. acidophilus* isolates. Across the 14 LAB species examined, the study identified distinct 25 RAPD profile types, based on 75% profile similarity (Table 2.1). Generally, there was high intra-specific RAPD profile variation. *L. casei*, *L. gasseri*, *L. jensenii, L. plantarum, L. suntoryeus* and *L. rhamnosus* all generated at least two distinct RAPD profiles per species, although the 12 isolates identified as *L. suntoryeus* showed only two RAPD profile types. The high level of sub-species diversity is consistent with levels seen in other studies on *L. plantarum* (Aznar and Chenoll, 2006), *L. rhamnosus* (Vancanneyt et al., 2006), *L. casei* (Roy et al., 1999) and *L. gasseri* (Du Plessis and Dicks,

1995). *L suntoryeus* is somewhat of a special case, as its 16S rRNA gene is 99.3% similar to *L. helveticus* (Cachat and Priest, 2005). Since the proposal of *L. suntoryeus* as novel species, sequence similarity analyses of housekeeping genes (*atpA, rpoA, pheS, tuf, slp* and *groEL*) have shown high relatedness between it and *L. helveticus,* leading to designation of *L. suntoryeus* as taxonomic unit with the species *L. helveticus* (Naser et al., 2006). , The two taxonomic groups could be clearly distinguished, however, by Amplified Fragment Length Polymorphism (AFLP) (Naser et al., 2006). In the current study two RAPD profile type were captured for isolates designated as *L. suntoryeus*, correlating to the reclassification of *L. helveticus* and identification of at least two taxonimic groups within this species (Naser et al., 2006).

2.4.1.2. Typing *L. acidophilus*

Despite numerous studies detailing the application of RAPD strain typing to other LAB species (section 2.4.1.1) there has been little work to establish the diversity of *L. acidophilus* at the strain level, despite its broad commercial importance. Numerous studies have evaluated the interspecific diversity of the *L. acidophilus* group (Johnson et al., 1980, Berger et al., 2007, Schillinger et al., 2003), but this is the first to examine such a number of strictly *L. acidophilus* species isolates in the context of other LAB. In contrast to the relatively high level of intra-specific RAPD profile diversity of the other LAB isolates tested, 10 reference, 15 commercial probiotic and 11 other isolates of *L. acidophilus* were assigned the same RAPD type (type 1). The reference isolates were originally deposited into culture collections in disparate years (Figure 2.2) and were originally isolated from various hosts and systems (Table 2.1), including rat faeces (*L. acidophilus* LMG 11428), humans (*L. acidophilus* LMG 9433). When expanded to cover isolates from industrial applications such as in probiotic products (*L. acidophilus* CUL21 and *L. acidophilus* CUL60), the current study has arguably one of the widest scopes for capturing strain diversity within this species.

Although RAPD data should be used only tentatively to describe genetic relatedness between isolates of LAB, this study also demonstrated the power of RAPD as a strain typing method across multiple LAB. With this fact in mind, since this study demonstrated that the numerous isolations of *L. acidophilus* (disparate both geographically and temporally), all had the same RAPD profile type, it suggests that either: (i) *L. acidophilus* may be a monophyletic *Lactobacillus* taxon, (ii) that the examined isolates belonged to the same evolutionary clade, or (iii) the isolates examined do not fully represent the diversity of *L. acidophilus* strains. To examine the latter question of whether full isolate diversity had been captured, an accurate cultivation-independent method of identifying *L. acidophilus* using the PAU-region was devised in order to rapidly identify whether it was present in other hosts, such as wild rodents. If *L. acidophilus* DNA was detected in these faecal samples, then a cultivation-based approach could be applied to collect these isolates and add further diversity to the isolate collections available for RAPD genotyping analysis.

2.4.2. PAU REGIONS AS SPECIFIC MARKER REGIONS FOR *L. ACIDOPHILUS*

Previous studies have developed PCR primers or specific oligonucleotide probes for *L. acidophilus* targeting the 23S rRNA gene (Pot et al., 1993), the ribosomal intergenic spacer (ITS) region (Song et al., 2000) and the 16S rRNA gene (Walter et al., 2000). The sequence identity observed in the 16S rRNA gene of the *L. acidophilus* group which is >99% in between certain species (Sarmiento-Rubiano et al., 2010) makes it a challenging target for species-specific probes design. This high level of identity may also account for the reduced specificity of probes based on the rDNA region and raises the question of developing marker regions from other genomic loci would prove a more accurate means to differentiate between *Lactobacillus* species.

A simple PCR test for the presence of PAU3 was developed with the design of PCR primers to amplify a region of an ORF present in PAU3 (Figure 2.3, Table 2.3). As *L. acidophilus* NCFM is the only complete genome sequence available, this study screened 32 lactobacilli (25 *L. acidophilus* isolates) to examine the distribution of PAU3 among lactobacilli and hence validate the region as one specific to *L. acidophilus*. All but two

isolates (LMG 11466 and LMG 11469) designated as *L. acidophilus* tested positive for the PAU3 specific marker (confirmed by DNA sequence), and no other lactobacilli (including members of the *L. acidophilus* group) cross-reacted with the specific PCR primers. The lack of the PAU3 marker region in *L. acidophilus* LMG 11466 and LMG 11467 was attributed to an absence of this DNA after genome re-sequencing (see Chapter 4) PAU regions are phage remnants and instability is associated with these mobile elements (Altermann et al., 2005, Brüssow, 2001). Further discussion of the genomics of this region is provided in Chapter 4.

The PAU region of the *L. acidophilus* NCFM genome was originally selected as a potential *L. acidophilus* specific target region based on its highly conserved sequence within genome sequenced *L. acidophilus* isolates and because it was unique in sequence to *L. acidophilus* isolates when tested with a nucleotide Blast (BlastN). Recent analyses have shown however, that PAU region structural homologues exist in *L. johnsonii* NCC533, *Lactococcus lactis* subsp. *cremoris* SK11 and *Streptococcus agalacticae* NEM316 (Altermann and Klaenhammer, 2011). The amino acid sequences of these regions are less conserved between species than they are within the same genome, that is to say PAU3 is more similar to PAU2 and PAU1in *L. acidophilus* than it is to any PAU analogues in other species, although functional classification of the genes in these regions is conserved, suggesting that these elements arose from different roots (Altermann and Klaenhammer, 2011). The wider than anticipated distribution of these elements, which combine characteristics of both bacteriophage and plasmid DNA, suggests that they are not peculiar to the *L. acidophilus* genome, but potentially represent a whole class of mobile genetic elements that may be sub-divided into distinct families. No function or *in vivo* activity has yet been attributed to these regions (Altermann and Klaenhammer, 2011). Despite the presence of the these structural homologues in other LAB species, the LBA479 ORF probes developed to detect the *L. acidophilus* PAU3 remain 100% specific for this region as the PCR primers in terms of sequence homology and are mis-matched in these other PAU homologous regions.

2.4.3. HOST RANGE OF *L. ACIDOPHILUS*

2.4.3.1. Searching for *L. acidophilus* in wild rodents

The detection limit of the specific marker test improved to 50 cfu by developing a nested PCR primer set, combined with a two-round PCR thermal cycling program. When applied to total DNA extracted from wild rodent faeces, no PAU DNA was detected, indicating an absence, or at least undetectable levels of *L. acidophilus* present. Culture-independent studies have shown that lactobacilli represent between 5 and 8% of the total sequence reads from the natural rodent gut, however their high species-level diversity would cause the abundance of a single species to be relatively low (Brooks et al., 2003, Tomas et al., 2012). When applied to rodents that were being fed the Lab4® probiotic, whose proportional presence of *L. acidophilus* was expected to be higher as they had been fed over 10^9 *L. acidophilus* each day, the PAU-based specific marker system was able to detect *L. acidophilus* in 91% of rat faeces.

Using a culture-based approach, no *L. acidophilus* was isolated from the faeces of wild rodents. Proportionally large numbers of *L. animalis* and *L. murinus* were isolated, which was to be expected as both naturally reside in the gut of rodents (Fraga et al., 2005, Sarma-Rupavtarm et al., 2004). RAPD profile similarity analysis also showed no isolates with similar profiles to *L. acidophilus*, ensuring that the PAU-based *L. acidophilus* specific marker did not fail to identify any *L. acidophilus* that may have been present. Rodents such as rats and mice are commonly used to model systems of human disease, and metagenomic studies of rodents with perturbations of their normal gut microbiota abound (Turnbaugh et al., 2006, Murphy et al., 2010, Turnbaugh et al., 2008). These studies are performed on laboratory-bred and raised animals that certainly do not have a "wild type" microbiota.

L. acidophilus is well documented as a human associated organism with a human gut-specific gene set, functionally different to that of *Lactobacillus helveticus*, a species found in dairy products that shares 98.4% 16S rRNA sequence identity (O'Sullivan et al., 2009).

This study was able to detect *L. acidophilus* in human faeces in a semi-quantitative culture-independent manner, with between 32 and 75% of studied individuals appearing to carry *L. acidophilus* at a level over the lower detection limit of the specific marker test. This wide range of carriage rates of *L. acidophilus* in humans is reflected by what we already know about the "patchy" distribution of *L. acidophilus* in healthy adults from culture-based (Mahenthiralingam et al., 2009) and culture-independent studies (Kleerebezem and Vaughan, 2009, Walter, 2008). It should be noted however, that use of the 16S rRNA gene as molecular target for indexing microbial diversity in the GIT may give a broad overview; it is not effective at distinguishing some *Lactobacillus* to the species level because of the high 16S rRNA gene similarity. The development of next-generation sequencing technologies promising increased sequence read length at no detriment to overall diversity indexed will allow improved species-level assignments based solely on the extra phylogenetic information afforded by increased read lengths.

2.5. CONCLUSIONS

The main conclusions from this chapter are as follows:

1) RAPD profile similarity analysis is an effective method of both identifying LAB and examining their sub-species level genetic diversity. It was highly reproducible and could be successfully applied to a large collection of LAB isolates.

2) LAB generally show a relatively high amount of sub-species level diversity. The exceptions to this rule are *L. suntoryeus-L. helveticus* and *L. acidophilus*. *L. acidophilus* isolates were from disparate isolation locations, deposition dates and host species. All *L. acidophilus sensu stricto* isolates generated a single RAPD profile type, based on 75% profile similarity, indicating the possibility that *L. acidophilus* isolates were all from a single strain type.

3) To ensure that the genetic diversity of *L. acidophilus* was reflected by the isolates tested, wild rodents were surveyed for the presence of *L. acidophilus* both culture dependently and independently. The PAU-based specific marker test proved effective at detecting *L. acidophilus* specifically. No *L. acidophilus* was found in wild rodents and the human carriage rate was variable.

4) Further work, at a discriminatory power above that of RAPD, is necessary study to examine the genetic structure of *L. acidophilus* as a species and whether it is clonally monophyletic.

3. MINIMUM TAXONOMIC CRITERIA FOR BACTERIAL GENOME SEQUENCE DEPOSITIONS

The majority of the analyses and discussion within this chapter was published within:

"BULL, M. J., MARCHESI, J. R., VANDAMME, P., PLUMMER, S. & MAHENTHIRALINGAM, E. 2012. Minimum taxonomic criteria for bacterial genome sequence depositions and announcements. Journal of Microbiological Methods, 89, 18-21"

3.1. INTRODUCTION

The genomic revolution has impacted multiple fields of microbiology facilitating the application of DNA sequence-based analyses to numerous questions in ecology, infection and industry (Medini et al., 2008). Bacterial genome sequence information is highly accessible and with minimal training researchers that are not specialists in a given field can use it to develop a wealth of molecular tools such as strain or species-specific PCR markers. In doing so, however, researchers are heavily reliant on the accurate deposition of data associated with genome sequences and the genetic databases. Although the taxonomy of many bacterial groups continues to change, the use of correct nomenclature for bacterial species is part of a fundamental language that allows microbiologists to communicate with each other and across other disciplines. Hence, when the species nomenclature associated with a bacterial genome sequence is incorrect it can have broad implications and impact on a multitude of fields. In 2008 (Field et al.) recognised the need for minimum descriptive criteria for genomes and metagenomes. They provided examples of data deposition records listing multiple criteria that included taxonomic status as a leading descriptor (Claesson et al., 2008). However, correctly assigning taxonomic status to an organism's genome is generally left to the research group submitting the sequence. With the current level of sequence data being deposited, it is

difficult for the DNA databases to further analyse the sequences and ensure that the taxonomic status of a genome is correct.

The availability of a complete bacterial genome sequence facilitates the application of several bioinformatic analyses to enable the source organism to be assigned to formally classified species or phylogenetic groups (Coenye et al., 2005). For all newly determined bacterial genomes it should therefore be relatively straightforward to systematically classify the bacterium from which the genome originates. However, the deposition of genome sequences assigned to the wrong systematic nomenclature may still occur if the sequences are not carefully analysed in a taxonomic context. Oh *et al*. (2011) recently deposited a genome sequence for *Lactobacillus acidophilus* 30SC, a bacterial isolate recovered from swine gut. The taxonomy of the genus *Lactobacillus* has changed considerably in recent years rendering biochemical or phenotypic analysis alone unable to permit accurate species identification for several constituent groups. For the existing *Lactobacillus* species however, DNA sequence-based methods can facilitate their accurate assignment at the species level (Naser et al., 2007). By examination of the *L. acidophilus* 30SC genome using just two defining characteristics, its full length 16S rRNA gene phylogeny and two conserved protein-encoding gene phylogenies, we were able to demonstrate that the genome sequence in fact must have originated from a strain of *Lactobacillus amylovorus.* To reduce the potential for such misclassification in future, we outline a simple bioinformatic analysis scheme and a minimum set of taxonomic criteria that should be applied to bacterial genomes before their formal deposition and announcement to the microbiology community.

3.1.1. SPECIFIC AIMS

The aims of this chapter were as follows:

1) Analyse the phylogenetic placement of *L. acidophilus* 30SC using its recently published genome sequence.

2) Construct *Lactobacillus* phylogenies using straightforward analyses based on the 16S rRNA gene sequence.

3) Construct *Lactobacillus* phylogenies using analyses based on the sequence of other phylogenetic marker genes such as gyrase B.

4) Set out a standard bioinformatic process that can be applied to newly generated genome sequences to ensure that they are accurately assigned to the genus or species level before deposition to a database and subsequent announcement.

3.2. RESULTS

The methods used in this chapter are described below, not in the earlier methods chapter, as they form an integral part of the bioinformatic workflow that was developed to correctly assign taxonomy for a bacterial genome sequence.

3.2.1. METHODS REQUIRED TO IMPLEMENT THE BIOINFORMATIC WORKFLOW

3.2.1.1. *L. acidophilus* 30SC gene sequences

Full-length sequences of the 16S rRNA, *gyrB* and *pheS* genes were downloaded from the 30SC genome (GenBank ID: CP002559).

3.2.1.2. 16S rRNA gene systematics

The 16S rRNA gene sequence of *L. acidophilus* 30SC was compared to the Ribosomal Database Project II (RDP II; http://rdp.cme.msu.edu) databases using the sequence match (SeqMatch) tool to facilitate identification (Cole et al., 2009). The SeqMatch tool allowed the identification and subsequent acquisition of 16S rRNA gene sequences of 20 type strains that were most closely related to the 16S rRNA gene of *L. acidophilus* 30SC. These were imported into MEGA5 (Oh et al., 2011, Tamura et al., 2011), aligned and trimmed. MEGA5 was also used to construct a neighbour-joining phylogeny.

3.2.1.3. Protein-coding gene phylogenies

The gyrase B subunit gene, *gyrB,* sequences were acquired using the Functional Gene pipeline and repository (FunGene; http://fungene.cme.msu.edu); *gyrB* sequences from *Lactobacillus* type strains with 16S rRNA genes most closely related to *L. acidophilus* 30SC were specifically selected. Sequences of the phenylalanyl-tRNA synthase alpha subunit gene, *pheS,* were drawn from a study by Naser *et al* (2007) and downloaded from Genbank. The

gyrB and *pheS* were imported into MEGA5 and analysed in the same way as the 16S rRNA gene sequences.

3.2.2. A SIMPLE BIOINFORMATIC WORKFLOW FOR CLASSIFICATION OF BACTERIAL GENOMES

A bioinformatic scheme to facilitate the accurate taxonomic identification of a bacterial genome was developed (Figure 3.1). The workflow facilitated the assignment of genus- or species-level nomenclature in the case of known species, while for bacteria belonging to novel taxonomic groups it can be used to define a nearest phylogenetic neighbour. Since multiple bioinformatic analyses may be used to assign the taxonomy of a bacterial genome (Coenye et al., 2005), a simplified workflow dependent on just two phylogenetic criteria was selected. The scheme can be easily applied by researchers not necessarily skilled in bioinformatic analyses and despite its simplicity was sufficient to provide accurate resolution of a genome to a known species or taxonomic group.

The first analytical criterion analysed the 16S rRNA gene sequence of a genome (Figure 3.1) as the most fundamental genetic tool available for bacterial taxonomic classification (Stackebrandt and Goebel, 1994). Bacterial 16S rRNA gene sequences can be rapidly compared at the Ribosomal Database Project II (RDP II; http://rdp.cme.msu.edu) or other curated databases and the search criteria limited to only identify closely related sequences obtained from well classified type strains. If related species type strains are not available for a given genome sequence, the related 16S rRNA gene sequences from the RDP II output may be searched for reference strains or well characterized sequences for uncultured microorganisms; these organisms can then be identified as the nearest defined phylogenetic neighbours of the genome sequence (Figure 3.1).

The second taxonomic criterion applied in the bioinformatic workflow was to analyse the sequence of protein coding genes from the genome and compare them to homologs encoded

within the related species/phylogenetic groups revealed by the initial 16S rRNA gene analysis (Figure 3.1). Analysis of protein-coding genes such as *recA* (Eisen, 1995) or *gyrB* (Yamamoto and Harayama, 1996), has been shown to be highly effective in assigning the taxonomy of species with particularly conserved 16S rRNA gene sequences. However, since protein-coding gene phylogenies may not always be congruent with the 16S rRNA gene for many bacterial species due to the lower taxonomic resolution of the latter ribosomal gene and the possibility of recombination and lateral gene transfer (Lukjancenko et al., 2011), we suggest two or more protein-coding genes should be examined to bring a finer resolution to the taxonomic placement of a genome (Figure 3.1).

In addition to using the latter databases to compare the 16S rRNA gene and selected protein-coding gene, phylogenetic trees of both taxonomic markers should also be constructed using the sequences from the most closely related species (Figure 3.1). This step will ensure that an accurate evolutionary placement of genome can be made and avoid assigning relatedness based on the percentage match of DNA sequences. Examination of the 16S rRNA gene and protein-coding gene phylogenies should also be made to ensure they are consistent in their assignment of the nearest match to the genome. On the basis of these combined analyses the correct up-to-date bacterial nomenclature can be obtained from the List of Prokaryotic names with Standing in Nomenclature (LSPN; http://www.bacterio.cict.fr). If the genome does not match a validly named species in terms of its 16S rRNA or protein-coding gene analyses, its nearest well characterized phylogenetic neighbour should be provided (Figure 3.1).

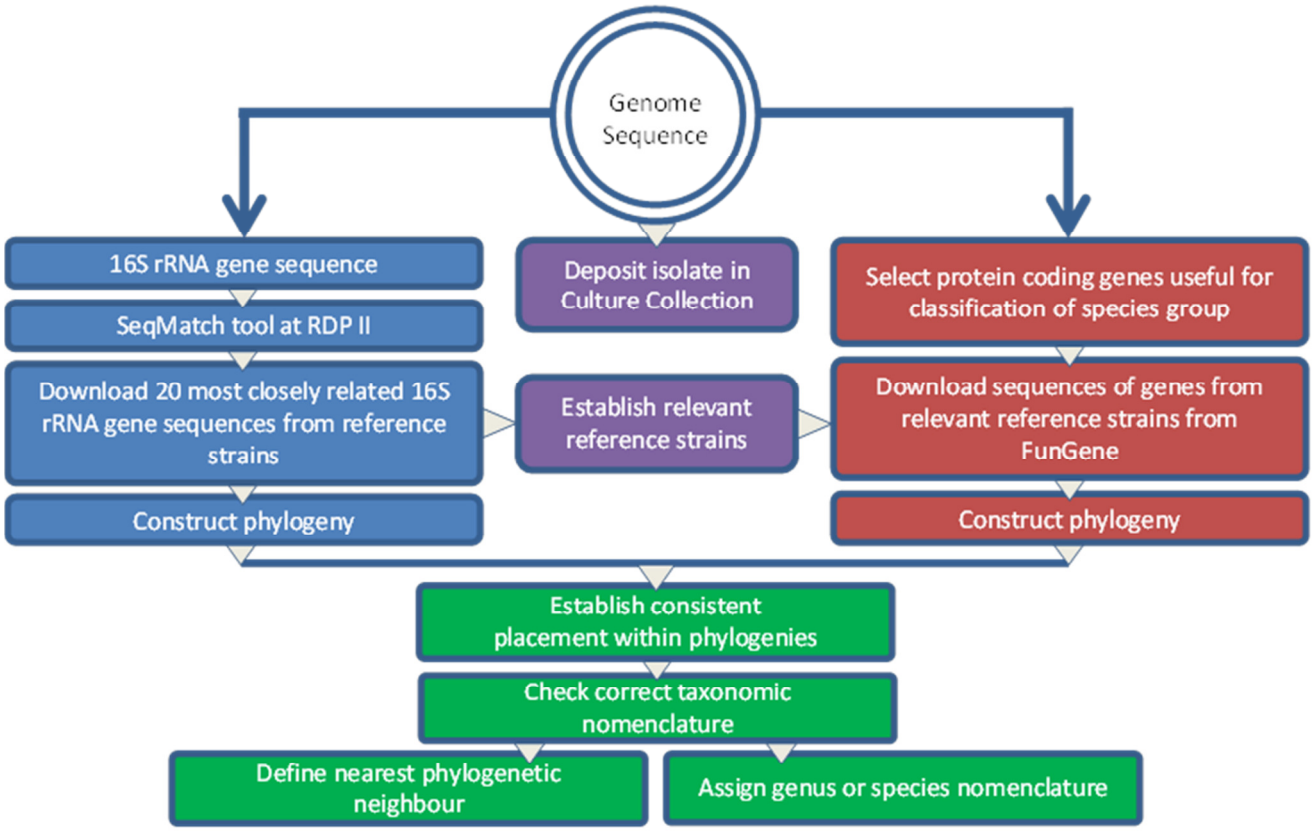


Figure 3.1: Bioinformatic work flow for assignment of taxonomic status to a bacterial genome.

An analysis scheme based primarily on analysis of the 16S rRNA gene with additional analysis of at least two protein coding genes is illustrated. The phylogeny of the latter genes should be compared for a given bacterial genome, and then the correct taxonomic nomenclature or nearest phylogenetic neigbor assigned. If the genome is representative of a cultured bacterial species, it should also be deposited in a recognised culture collection. The bioinformatic tools are available from the databases described in section 3.2.1.

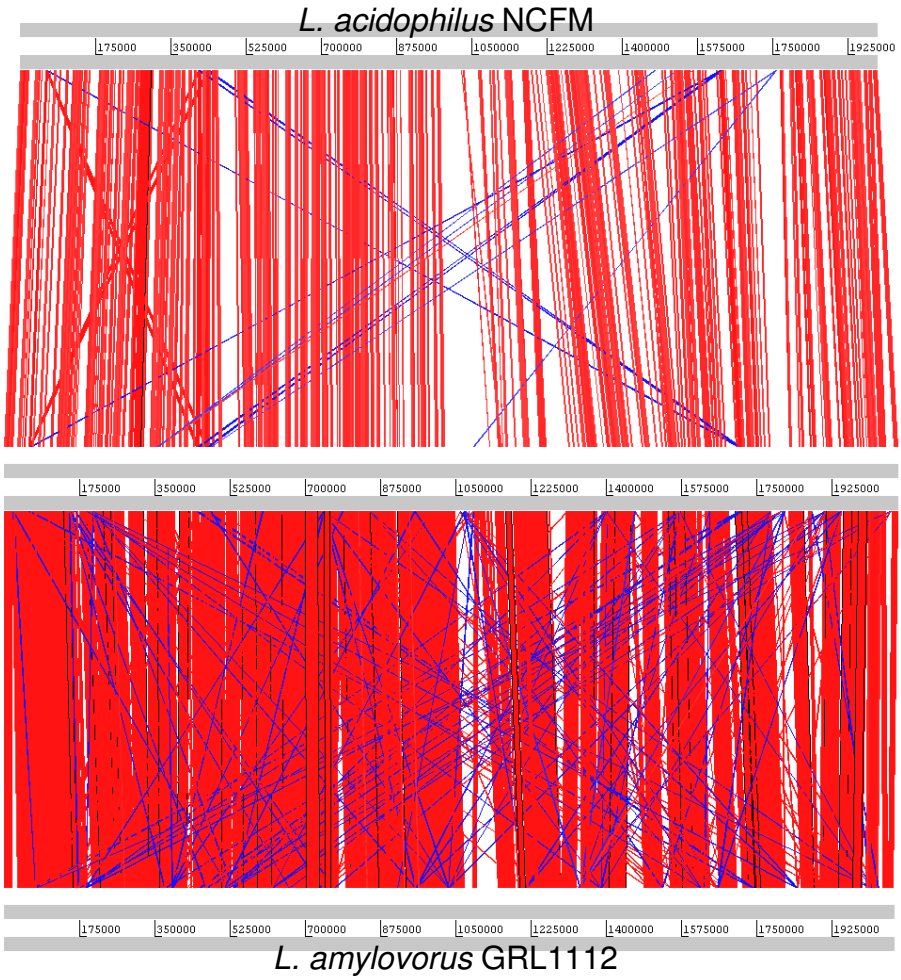


Figure 3.2: Whole genome comparison of *L. acidophilus* 30SC to *L. acidophilus* NCFM and *L. amylovorus* GRL1118.

Genome sequences are represented by horizontal lines with *L. acidophilus* as the central horizontal lines. Regions of similar sequence are linked with lines between genomes. Sequence identity is indicated by shade intensity. Red indicates similar sequence in the forward direction, blue in the reverse.

3.2.3. APPLICATION OF THE BIOINFORMATIC WORKFLOW TO THE *L. ACIDOPHILUS* 30SC GENOME

After deposition of the *L. acidophilus* 30SC genome, we performed a genome comparison to that of *L. acidophilus* NCFM, a well characterized probiotic strain of this species (Altermann et al., 2005), and observed that the two genomes were not closely related when compared using the Artemis Comparison Tool (ACT; Figure 3.2). The *L. acidophilus* 30SC genome sequence clearly showed greater identity with the genome sequence of *L. amylovorus* UCC1118 (indicated by the deeper shade of red) than the genome sequence of *L. acidophilus* NCFM (lighter shade of red). We therefore applied the bioinformatic analysis scheme and minimum criteria (Figure 3.1) to *L. acidophilus* 30SC genome to clarify its taxonomic assignment. Use of the SeqMatch tool at the RDP II demonstrated that the *L. acidophilus* 30SC 16S rRNA gene was most closely related to *L. amylovorus* sequences. After downloading and phylogenetically analysing the full length 16S rRNA genes for 20 type strains of the most closely related *Lactobacillus* species, the resulting tree also demonstrated that *L. acidophilus* 30SC sequence was most similar to that of *L. amylovorus* LMG 9496^T (Figure 3.3, panel A; 99.8% identity). The 16S rRNA gene for the *L. acidophilus*-type strain, LMG 9433^T, placed in a completely distinct phylogenetic cluster (Figure 3.3, panel A; 98.2% identity).

Since the taxonomy of genus *Lactobacillus* has been heavily revised in recent years by the description of numerous new species and because the discriminatory power of the 16S rRNA gene for differentiation of its constituent taxa is limited for several clusters of species, we searched the available literature to identify protein-coding genes which were useful for species identification. The *gyrB* gene had been used as part of a multilocus sequence typing (MLST) scheme for *Lactobacillus plantarum* strains (de las Rivas et al., 2006). We therefore used the FunGene database to compare the *gyrB* from the *L. acidophilus* 30SC genome to the *gyrB* genes available for type strains of those *Lactobacillus* species that were most closely related by analysis of the 16S rRNA gene (Figure 3.3, panel A). The *gyrB* phylogeny demonstrated that the *L. acidophilus* 30SC *gyrB* sequence was most similar to that of *L. amylovorus* LMG 9496^T (Figure 3.3, panel B; 99.8% identity). The *gyrB* sequence of the *L.*

acidophilus type strain, LMG 9433^T, clustered separately from that of strain 30SC (Figure 3.3, panel A) and was considerably less similar (88.1% identity).

Although the *gyrB* analysis was consistent with the 16S rRNA gene assignment, in order to avoid over reliance on a single-protein gene we searched the literature for an additional protein-coding gene useful for *Lactobacillus* systematics. Naser et al. (Naser et al., 2007) had examined the *pheS* gene and demonstrated that it offered a discriminatory means of species identification within the genus *Lactobacillus.* The *pheS* gene of the *L. acidophilus* 30SC genome was 99.1% similar to that of *L. amylovorus* and phylogenetically clustered with this species (Figure 3.4); it was not closely related to the *L. acidophilus pheS* sequence (Figure 3.4, 88.7% identity). Overall, even though the *gyrB* (Figure 3.3, panel B) and *pheS* (Figure 3.4) phylogenetic trees were not absolutely congruent with 16S rRNA gene phylogeny across all the *Lactobacillus* species examined, the respective *L. acidophilus* 30SC sequences were consistently placed adjacent *L. amylovorus* as the nearest phylogenetic neighbour and indicating this species is the most likely taxonomic group to which the 30SC isolate belonged.

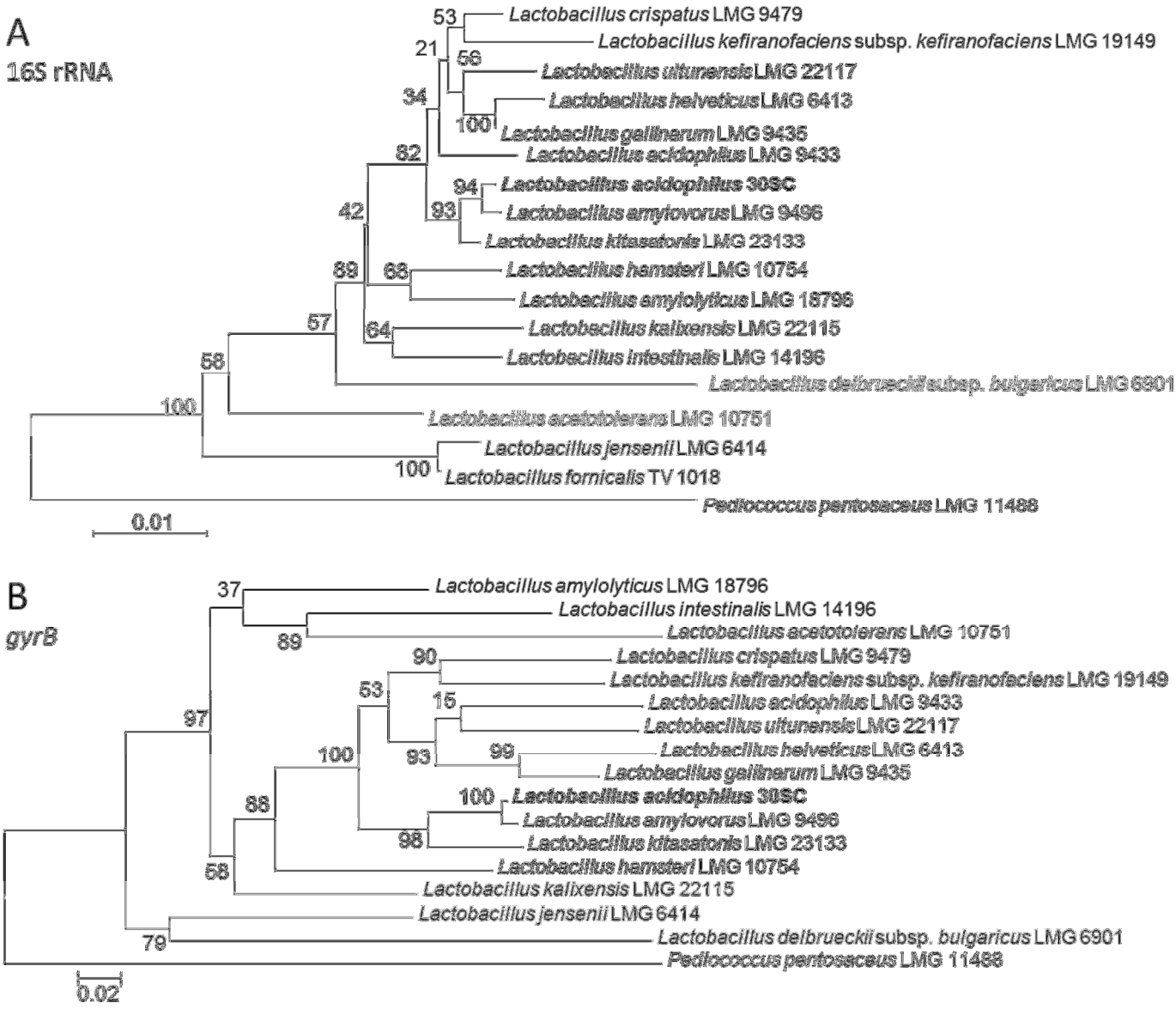


Figure 3.3: Phylogenetic trees of lactobacilli related to *L. acidophilus* 30SC.

Phylogenetic analysis of aligned 16S rRNA (panel A) and gyrB (panel B) genes from representative *Lactobacillus* reference strains classified as most closely related (similarity scores >0.949) to the *L. acidophilus* 30SC (indicated in bold font) genes is shown. The trees for each gene were rooted with the corresponding sequence from *Pediococcus pentosaceus* LMG 11488; the genetic distance scale and bootstrap values are indicated.

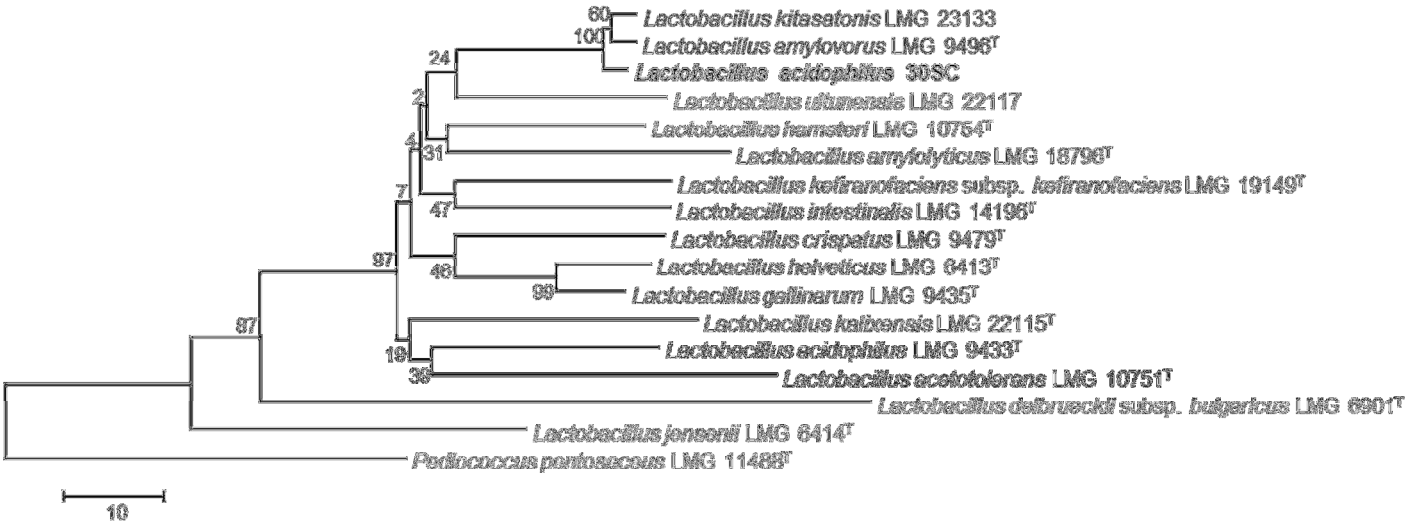


Figure 3.4: Phylogenetic tree of *pheS* genes of lactobacilli related to *L. acidophilus* 30SC

Phylogenetic analysis of aligned *pheS* genes from representative *Lactobacillus* reference strains classified as most closely related to the *L. acidophilus* 30SC (indicated in bold font) gene is shown. The tree was rooted with the corresponding sequence from *Pediococcus pentosaceus* LMG 11488; the genetic distance scale and bootstrap values are indicated.

3.3. DISCUSSION

With the continued improvement and innovation in technology, particularly the advent of next-generation sequencers, researchers have unprecedented access to nucleotide sequence data. There is no doubt that the massive expansion of DNA sequence datasets has considerably advanced the study of life sciences, however, there is also a feeling that our ability to collect sequence data far surpasses our ability and power to analyse it (Brenner, 2010). In addition, as more sequence analysis tools are developed to enable large scale data mining, the outputs from these analyses may have less value if the original sequence data inputs are poorly characterised at source. Here we illustrate an example of how such oversights are still occurring in genomic microbiology, with the taxonomically incorrect deposition and announcement of the *L. acidophilus* 30SC genome (Oh et al., 2011). Using a straightforward analysis based on two minimal criteria, assignment via the 16S rRNA gene and protein-coding gene phylogenies; we have clearly demonstrated that the *L. acidophilus* 30SC genome most likely derives from a strain of *L. amylovorus* (Figure 3.3).

Many disciplines rely heavily on taxonomic nomenclature to provide a common language that can be understood by both specialist researchers and also extend into wider public understanding. Taxonomy is particularly important in microbiology where there is such an extensive diversity of organisms, that a microbial commons and systematic guidelines are absolutely vital for advancement of the discipline (Moore et al., 2010). Like many microorganisms, the lactic acid bacteria, within which *L. amylovorus* and *L. acidophilus* reside, are of considerable ecological, clinical and commercial interest (Pfeiler and Klaenhammer, 2007). The widespread use of these bacteria as probiotics and diary starter cultures forms a multi-million dollar industry, with basic researchers, industry and regulatory agencies demanding much clearer definitions of *Lactobacillus* strains. The incorrect deposition of the strain 30SC genome as a representative of the species *L. acidophilus* may potentially have had significant future ramifications, especially since genomes for these bacteria are now being obtained by non-specialist researchers and commercial groups.

In summary, we suggest the need for a systematic review of the way genome sequence data is deposited and propose a simple, minimum-standard system for characterisation of new bacterial genome sequences prior to their announcement. We have proposed an analytical scheme which is straightforward and for the most part can be performed using publicly available databases and software, to compare the 16S rRNA gene and at least two protein-coding genes from a given genome. For researchers skilled in bioinformatic analyses, this scheme could easily be expanded to include analyses of multiple protein coding genes such as those used in MLST schemes (de las Rivas et al., 2006), the average nucleotide identity of shared genes or even whole genome phylogenies (Konstantinidis and Tiedje, 2005). In addition, we also suggest that if the genome sequence is for an easily cultured microorganism, that the corresponding strain is deposited in a recognised International Depository Authority culture collection and hence can be easily analysed by the research community (Figure 3.3); this will add considerable future value to a microbial genome sequence. We hope that our illustration of genome misclassification and a simple bioinformatic workflow to avoid it will increase the consistency of future genome sequence taxonomy. This will ensure that users of these incredibly valuable genomic datasets, particularly those who are not specialists in the field, can be confident in the identity of a deposited sequence.

3.4. CONCLUSIONS

The conclusions from this chapter are as follows:

1) The developed bioinformatic workflow successfully identified that the *L. acidophilus* 30SC genome was wrongly attributed to *L. acidophilus.* DNA biomarker genes used for this bioinformatics workflow, the 16S rRNA, *gyrB* and *pheS* genes, all showed greater similarity to *L. amylovorus* LMG 9496. Secondarily, whole genome comparisons of available *L. acidophilus* and *L. amylovorus* genome sequences also showed that the genome of *L. acidophilus* 30SC showed greater synteny with *L. amylovorus* UCC1118 than *L. acidophilus* NCFM.

2) The reliance of future analyses on the attached metadata of genome sequence depositions means that it is imperative that this metadata is correct, particularly the species name that is attached to the genome record. The proliferation of genome resequencing means that a technology once restricted to the cutting-edge of genomics is now available to a wider audience, including non-specialists and commercial parties. The development of a simple bioinformatic workflow for classifying a genome sequence will ensure that users of a deposited sequence can be confident of its identity and nomenclature.

3) Organisms of considerable ecological, clinical and commercial interest, like *L. acidophilus* and *L. amylovorus,* may have probiotic effects that are strain-specific. Therefore, ensuring that a genome sequence for one of these bacteria is assigned the correct taxonomic nomenclature is vitally important. In addition, the regulatory standards for including bacteria in probiotic and food-related products continue to be developed, with accurate descriptions of the organisms present now a fundamental criterion.

Note added in proof:

Analysis of glycolysis and pentose phosphate pathways across *Lactobacillales* to depict evolution of this order recently corroborated the phylogenetic placement and reclassification of *L. acidophilus* 30SC. Salvetti *et al* (2013) used genome sequences from 42 LAB and inferred phylogeny using the concatenated sequences of 42 ribosomal proteins. Subsequently, the distribution and organisation of 42 genes related to glycolysis and the pentose phosphate pathway was analysed. *L. acidophilus* 30SC was identical to *L. amylovorus* GRL1112 at all ribosomal sites and all glycolysis pathway loci. Interestingly, *L. acidophilus* 30SC differed to *L. amylovorus* GRL1112 at only a single locus of its pentose phosphate pathway.

4. POPULATION GENOMICS OF *L. ACIDOPHILUS*

4.1. INTRODUCTION

4.1.1. GENOMICS IN LAB

Numerous approaches have been used to explore the relationships and species assignments of members of the LAB (see Section 1.3.1). These include DNA-DNA hybridization, 16S rRNA gene sequence analysis, phenotypic characteristics and sequence analysis of other phylogenetic marker genes. These analyses have ranged in taxonomic breadth from examining all LAB (Makarova et al., 2006, O'Sullivan et al., 2009), to all lactobacilli (Claesson et al., 2008), to the *L. acidophilus* group (Berger et al., 2007), to strains of a single *Lactobacillus* species (Hao et al., 2011a). The evolution of techniques for identifying and classifying bacteria has impacted on the taxonomy of the LAB, resulting in numerous species re-classifications and re-definitions of above-species-level taxonomic groups (Salvetti et al., 2012).

The recent availability of whole genome sequences of probiotic bacteria, and the reduction in financial investment and expertise required to re-sequence new isolates for species that already have a representative genome-sequenced isolate, has made the use of genome sequences to characterise bacteria, a viable proposition for a growing number of the scientific community. Bacterial genome sequencing is now being carried out by a large number of research laboratories and commercial enterprises, to examine environmental and health-related issues.

Several post-genomic studies have been undertaken on various taxonomic levels within the LAB. These include the examination of phylogenetic relationships between bacteria on the i) genus level from both a phylogenetic (Makarova et al., 2006, Coenye and Vandamme, 2003) and functional (Klaenhammer et al., 2005, O'Sullivan et al., 2009) perspective, ii) group level (Berger et al., 2007) and iii) the species level in both

Lactococcus lactis (Passerini et al., 2010) and *L. plantarum* (Molenaar et al., 2005). No single study however, has fully utilised next-generation sequencing technologies in combination with highly efficient genome assembly and analysis methods to carry out genomic diversity profiling of a large cohort of isolates of a single LAB species. In this section, the genomic diversity of *L. acidophilus* was explored at multiple taxonomic levels. During the study, access to next-generation sequencing (NGS) technologies altered so much that two approaches were used. The first used single genome re-sequencing by a commercial sequence provider and illustrates how genome sequencing technologies have made single genome resequencing economically viable. The second approach used massively-parallel genome resequencing and assembly technologies provided by an academic genome sequencing centre, together with the analysis on a web-based database (Jolley and Maiden, 2010), and illustrates how decoding of tens of bacterial genomes is now a viable prospect for LAB researchers

4.1.2. APPLICATION OF NOVEL GENOME SURVEYING STRATEGIES

4.1.2.1. Analysing multiple bacterial genomes

The advent of massively parallel sequencing technologies has revolutionised phylogenetic data collection with massively multiplexed, high-throughput, short-read sequencing making it possible to obtain DNA sequence for virtually every locus of a genome in an achievable time-frame and at economical cost (Medini et al., 2008). With the decline in costs of genome sequencing, it is now more economical to derive an MLST profile (normally the partial sequencings of seven house-keeping gene loci) by sequencing the entire genome of an isolate and extracting the sequences of the relevant loci, than the classical method of amplifying each locus with specific PCR primers and Sanger sequencing the resulting product (Sheppard et al., 2012). The derivation of a complete genome sequence for isolates of interest undoubtedly provides a wide range of opportunities to improve the understanding of epidemiology and functional biology of bacterial populations, far beyond a simple MLST scheme. There are various approaches to

describe genome sequence variation among bacterial isolates, and these, together with other interesting variable loci which can be mined from the data are discussed below.

4.1.2.2. Analysis of genomes using a reference genome sequence

The mapping of sequence data from multiple isolates to a finished reference genome sequence allows the identification of polymorphic sites compared to this reference isolate. This can be accomplished with relative ease and is computationally, orders of magnitude less intensive than a *de novo* sequence assembly. Analysis of Single Nucleotide Polymorphisms (SNPs) generated by read mapping has been used to compare genomes of clinically important pathogens (Harris et al., 2010) and probiotic species (Briczinski et al., 2009). The results should however, be interpreted with some degree of caution. Parallel sequencing technologies are relatively more error prone than classical sequencing technologies, and read mappings will be highly dependent on the finished reference sequence to which variation is mapped. Therefore, the reference sequence used should be highly representative of the isolates that are being compared to it, as variation in genes that are not present in the reference genome cannot be detected

4.1.2.3. Analysis of genomes using gene-by-gene comparison to a reference sequence

An alternative to the reference genome sequence assembly approach is a *de novo*, reference-free assembly, followed by gene-by-gene comparisons to a reference sequence (Jolley and Maiden, 2010). The unit of comparison and analysis then becomes a more functional unit (i.e. the gene[s]), rather than the whole genome. In this case, "the gene" as a unit of comparison may be extended to include any identifiable unit of analysis, including but not limited to sequences found at a particular genetic locus, given coding sequences (CDS) or any other identifiable sequence motif whether it be nucleotide or protein (Sheppard et al., 2012). The variation between genomes is then categorised one "gene" unit at a time, essentially in the same way the variation between MLST loci is described (Maiden, 2006). Although MLST schemes to date are generally composed of seven loci there is no reason, other than balancing the economy of research investment with information return, that the scheme should not be extended to encompass more loci.

As the investment in generating a genome sequence assembly for a single isolate is now small enough to be a viable proposition for many researchers, MLST may now be extended to incorporate all loci defined within a particular reference genome sequence, as such creating a "genome-wide" MLST scheme (Sheppard et al., 2012).

There are several advantages to the gene-by-gene approach when compared to approaches that rely on multiple- or pairwise-alignment of whole genome sequences for the identification of SNPs (Sheppard et al., 2012). Comparisons are performed using "gene"-length units that can be compared to establish the total diversity in each one of these regions, removing the need for closely related reference sequences for mapping. This increases the scope of the approach to handle genomes from diverse sources. Secondarily, the gene-by-gene approach is particularly well-suited to analysing the output from *de novo* sequence assemblers, which often produce partial, draft genome sequences comprised of multiple contigs. Thirdly, any sequencing errors that are introduced by the parallel sequencing technology are screened out by ensuring that sequencing depth is great enough to identify them in the assembly process. The main limitation of the gene-by-gene approach is similar to that of the whole genome mapping approach, in that variation can only be captured in loci already defined in the reference gene set, so information outside of these regions will not be analysed, and the reference gene set will be expected to define the whole set of loci to be compared. Also, this technique relies heavily on the ability of the *de novo* assembly to construct draft genomes composed of a few, long contigs. The success of generating such contigs is linked to the genome sequence features of the isolates studied, with small, low complexity, low GC genomes ideal for this analysis. It is also highly dependent on the sequencing quality obtained and type of NGS technology applied.

4.1.2.4. CRISPR regions

A detailed discussion of CRISPR regions in *L. acidophilus* can be found in Chapter 1. CRISPR regions have been found in over half of the genome sequences available for lactobacilli, although they vary in sequence considerably across the genus *Lactobacillus*.

In *Escherichia coli*, CRISPR regions are highly variable, and can be used as strain specific markers (Díez-Villaseñor et al., 2010). Given that RAPD profile similarity analysis of *L. acidophilus* isolates shows limited diversity (Chapter 3), the CRISPR regions of this species investigated further as loci with the greatest chance of variation between highly clonal isolates, as seen in *B. animalis* subsp. *lactis* (Milani et al., 2013) .

4.1.2.5. Mutagenesis of *L. acidophilus*

The human GIT has been designated as the natural niche of *L. acidophilus* (O'Sullivan et al., 2009). The gut is known to be potentially mutagenic environment (Hirayama et al., 2000), with multiple metabolites, particularly those generating from anaerobic fermentation, capable of inducing genetic mutations. The stability of *L. acidophilus* RAPD profiles generated by isolates passaged through the human GIT (Mahenthiralingam et al., 2009) suggested that either the environment is not sufficiently mutagenic to induce sequence variation detectable at such a granular level, or that *L. acidophilus* is capable of efficiently repairing DNA damage and maintaining a highly stable genome. Exposure to the chemical mutagen Methyl Methanesulphonate (MMS) has been shown to induce mutations in wide range of bacteria, including mutations that result in detectable phenotypes in *Lactobacillus delbrueckii* ATCC 9649 (Demirci and Pometto, 1992), a member of the *L. acidophilus* taxonomic sub-group. Given the successful use of MMS in a closely related species, it was used to further investigate the susceptibility of *L. acidophilus* to the type of chemical mutation that might occur in the human GIT.

4.1.3. SPECIFIC AIMS

The aims of this chapter were as follows:

- Conduct a small-scale genome diversity survey using reference mapping of sequencing reads, combined with SNP discovery.

- Expand genomic information on *L. acidophilus* isolates by resequencing the genomes of a diverse collection of isolates from commercial applications and culture collections.

- Conduct a wider-scale genomic diversity study to establish evolutionary history of *L. acidophilus* isolates using a gene-by-gene comparative approach applied via rMLST at the genus-species level and genome-wide MLST at the species-strain level.

- Investigate the diversity of the CRISPR region of the *L. acidophilus* genome.

- Investigate mutability of *L. acidophilus* using Methyl Methane Sulphonate (MMS) to alkylate DNA and induce mutations, using genome resequencing and gene-by-gene comparisons to detect any DNA sequence changes that occurred.

4.2. METHODS

4.2.1. GENOME RESEQUENCING STRATEGIES

Two different genome resequencing strategies were employed, reflecting the development of Illumina sequencing technology. The first was applied on a small scale at higher cost, and the second was applied on a larger scale as follows.

4.2.1.1. Small-scale genome resequencing and comparison strategy

High molecular weight genomic DNA was extracted from the growth of single-colony overnight cultures with a Wizard genomic DNA purification kit (Promega, Southampton, United Kingdom). Genomic DNA libraries were prepared and single-reads were sequenced by GATC Biotech (Konstanz, Germany) using an Illumina HiSeq2000 with 50 bp sequence read length. Single reads were aligned to the *L. acidophilus* NCFM complete genome sequence (GenBank: CP00003) using Burrows-Wheeler Alignment Tool (bwa) (Li and Durbin, 2009), and a consensus sequence created. Consensus sequences were aligned with Mugsy (Angiuoli and Salzberg, 2011). Phylogenies were calculated using MEGA 5.1 (Tamura et al., 2011). Whole genome sequence similarity was visualised with the Artemis Comparison Tool (Carver et al., 2005).

4.2.1.2. Large-scale genome resequencing and comparison strategy

As previously, high molecular weight genomic DNA was extracted from the growth of single-colony overnight cultures with a Wizard genomic DNA purification kit (Promega, Southampton, United Kingdom). Genomic libraries were prepared and DNA sequenced at the Wellcome Trust Centre for Human Genetics, Oxford, UK (http://www.well.ox.ac.uk/ogc/home). All genomic libraries were pooled in equimolar amounts and analysed in a single flow cell lane of the Illumina HiSeq2000, generating 100 bp paired-end reads. At this stage, the 50 bp single-reads were introduced from the small-scale genome resequencing study along with 100 bp paired-end reads from two *L.*

acidophilus genomes (SRA refs: ERR203994 and ERR256998), downloaded from the Sequence Read Archive (SRA) at the NCBI.

Genome sequences were assembled using Velvet version 1.2.01 shuffle and optimisation scripts, creating contigs with optimal parameters, with *k*-mer lengths between 87 and 95 bp for the paired-end sequence reads generated as part of the wide-scale study, 45 and 49 bp for 50 bp single reads from the small-scale study, and 99bp for the sequence reads taken from the SRA (Zerbino, 2010, Cody et al., 2013) (Table 4.1). Assembled data were deposited in the PubMLST database as implemented by the Bacterial Isolate Genome Sequence Database (BIGSDB) software platform (Jolley and Maiden, 2010). To supplement and contextualise the new sequence information generated by this study, further completed and draft genome sequence data from the *L. acidophilus* group were downloaded from the Integrated Microbial Genomes (IMG) database (Markowitz et al., 2010) and also deposited into the PubMLST database (Table 4.2).

Table 4.1: Velvet assembly statistics of *Lactobacillus* isolate genomes

Species	Isolate	Number of sequence reads	Sequence Read Length	Contig N50	Number of Contigs >1Kb
Lactobacillus acidophilus	C21	10353702	100	182313	28
Lactobacillus acidophilus	C46	10776998	100	172985	29
Lactobacillus acidophilus	C47	8590468	100	182321	30
Lactobacillus acidophilus	C49	9788514	100	182321	29
Lactobacillus acidophilus	CUL 21	8648162	100	183130	30
Lactobacillus acidophilus	CUL 60	8372568	100	182851	25
Lactobacillus acidophilus	CulT2	6977356	100	183128	29
Lactobacillus acidophilus	HBCA	9716532	100	182325	25
Lactobacillus acidophilus	LAB 283	10694936	100	120702	28
Lactobacillus acidophilus	LAB 66	10941612	100	167481	33
Lactobacillus acidophilus	LAB 69	11762534	100	172941	30
Lactobacillus acidophilus	LAB 76	10244062	100	167485	33
Lactobacillus acidophilus	LMG 11428	11780070	100	81116	55
Lactobacillus acidophilus	LMG 11466	10754308	100	228858	24
Lactobacillus acidophilus	LMG 11467	12324202	100	242199	21
Lactobacillus acidophilus	LMG 11469	13525190	100	143813	31
Lactobacillus acidophilus	LMG 11470	12022574	100	167948	33
Lactobacillus acidophilus	LMG 11472	12067104	100	167526	34
Lactobacillus acidophilus	LMG 13550	11520676	100	173001	28
Lactobacillus acidophilus	LMG 9433	7833202	100	167519	29
Lactobacillus acidophilus	NCFM	8119384	100	174801	28
Lactobacillus acidophilus	NCFM 1-1	7787216	100	183130	26
Lactobacillus acidophilus	NCFM 1-2	10458212	100	167511	36
Lactobacillus acidophilus	NCFM 2-1	9941284	100	173994	30
Lactobacillus acidophilus	NCFM 2-4	8684056	100	182321	32
Lactobacillus acidophilus	NCFM 3-1	6974196	100	182317	31
Lactobacillus acidophilus	NCFM 3-2	9596090	100	182321	33
Lactobacillus acidophilus	Rm 344	9981982	100	184420	21
Lactobacillus acidophilus	Rm 345	13810300	100	183241	23
Lactobacillus acidophilus	CUL 60 S	18965596	51	81591	50
Lactobacillus acidophilus	LMG 11428 S	31352785	51	120621	42
Lactobacillus acidophilus	CUL 21 S	23488108	51	78118	48
Lactobacillus acidophilus	ERR203994	2601602	100	242509	24
Lactobacillus acidophilus	ERR256998	6433322	100	167515	23
Lactobacillus gasseri	LMG 9203	6505684	100	257847	14

Table 4.2: Genome sequences of *L. acidophilus* group isolates deposited in the PubMLST database for this study

Species	Isolate	Aliases	Country	Year	Isolate source	Sequence source	Genome status
L. acidophilus	NCFM	NCFM R	USA	1970	Derived from LMG 9433	NCBI RefSeq	finished
L. acidophilus	ATCC 4796	LMG 11470	USA	1980	Human Microbiome Project	NCBI RefSeq	draft
L. acidophilus	C21	-	UK	2008	Cultech isolate	This study	draft
L. acidophilus	C46	-	UK	2008	Cultech isolate	This study	draft
L. acidophilus	C47	-	UK	2008	Cultech isolate	This study	draft
L. acidophilus	C49	-	UK	2008	Cultech isolate	This study	draft
L. acidophilus	CUL 21	-	UK	2004	ProHEMI isolate	This study	draft
L. acidophilus	CUL 60	-	UK	2004	ProHEMI isolate	This study	draft
L. acidophilus	CulT2	-	UK	2008	Cultech isolate	This study	draft
L. acidophilus	HBCA	-	UK	2008	Isolated from product	This study	draft
L. acidophilus	LAB 283	ATCC 4357; Kulp strain PAK	USA	1963	-	This study	draft
L. acidophilus	LAB 66	LMG 11428	USA	1922	-	This study	draft
L. acidophilus	LAB 69	-		-	-	This study	draft
L. acidophilus	LAB 76	LMG 11428	USA	1922	-	This study	draft
L. acidophilus	LMG 11428	ATCC 832; Rettger 4B	USA	1922	Fed lab Rat	This study	draft
L. acidophilus	LMG 11466	-	UK	1960	NIRD	This study	draft
L. acidophilus	LMG 11467	ATCC 314; L. F. Rettger 43	USA	1920	Human	This study	draft
L. acidophilus	LMG 11469	ATCC 4355; Kulp R-1-1	USA	1924	Rat or human (Kulp & Rettger, 1924)	This study	draft
L. acidophilus	LMG 11470	ATCC 4796	USA	1980	-	This study	draft
L. acidophilus	LMG 11472	ATCC 9224	USA	1950	-	This study	draft
L. acidophilus	LMG 13550	LMG 9433; ATCC 4356	USA	1964	Human	This study	draft
L. acidophilus	LMG 9433	ATCC 4356; LMG 13550	USA	1964	Human	This study	draft
L. acidophilus	NCFM	Derived from LMG 9433	Canada	1975	-	This study	draft

Table 4.2: Genome sequences of *L. acidophilus* group isolates deposited in the PubMLST database for this study (cont.)

Species	Isolate	Aliases	Country	Year	Isolate source	Sequence source	Genome status
L. acidophilus	Rm 344	-	China	2012	Cultech isolate	This study	draft
L. acidophilus	Rm 345	-	China	2012	Cultech isolate	This study	draft
L. acidophilus	CUL 60 S	CUL 60	UK	2004		Small-scale *L. acidophilus* genome study (50bp single reads)	draft
L. acidophilus	LMG 11428 S	LMG 11428	USA	1922		Small-scale *L. acidophilus* genome study (50bp single reads)	draft
L. acidophilus	CUL 21 S	CUL 21	UK	2004		Small-scale *L. acidophilus* genome study (50bp single reads)	draft
L. acidophilus	ERR203994	LMG 9433; LMG 13550; ATCC 4356	USA	1964		NCBI Sequence read archive (SRA)	draft
L. acidophilus	ERR256998	-	-	-		NCBI Sequence read archive (SRA)	draft
L. crispatus	JV-V01	-	-	-	Human vaginal flora	NCBI RefSeq	draft
L. crispatus	MV-1A-US	-	-	-	Human vagina	NCBI RefSeq	draft
L. delbrueckii	ATCC 11842	-	Bulgaria	1919	Yoghurt	NCBI RefSeq	finished
L. delbrueckii	ATCC BAA-365	-	France	1987	Gastrointestinal	NCBI RefSeq	finished
L. gasseri	ATCC 33323	-	-	-		NCBI RefSeq	finished
L. gasseri	202-4	-	-	-		NCBI RefSeq	draft
L. gasseri	JV-V03	-	-	-	Human vaginal flora	NCBI RefSeq	draft
L. gasseri	LMG 9203	-	France	1970		This study	draft
L. helveticus	DSM 20075	-	-	-	Emmental cheese	NCBI RefSeq	draft
L. helveticus	DPC 4571	-	-	-		NCBI RefSeq	finished
L. iners	DSM 13335	-	Sweden	-	Urine	NCBI RefSeq	draft

Table 4.2: Genome sequences of *L. acidophilus* group isolates deposited in the PubMLST database for this study (cont.)

Species	Isolate	Aliases	Country	Year	Isolate source	Sequence source	Genome status
L. jensenii	269-3	-	-		Human vaginal cavity	NCBI RefSeq	draft
L. jensenii	JV-V16	-	USA		Human vagina	NCBI RefSeq	draft
L. jensenii	27-2-CHN	-	-		Human vagina	NCBI RefSeq	draft
L. jensenii	115-3-CHN	-	-		Human vagina	NCBI RefSeq	draft
L. johnsonii	FI9785	-	-		Human vagina	NCBI RefSeq	finished
L. johnsonii	ATCC 33200	-	Belgium		Human blood	NCBI RefSeq	draft
L. johnsonii	NCC 533	-	-		Faeces	NCBI RefSeq	finished
L. ultunensis	DSM16047	-	Sweden		Human	NCBI RefSeq	draft

Rows containing data relating to *L. acidophilus* records are coloured based on their history; commercial isolates in green and culture collection isolates in blue

4.2.2. IMPLEMENTATION OF GENE-BY-GENE ANALYSIS APPROACHES

4.2.2.1. Generation and storage of data using BIGSDB

The three main data components of this approach were generated as follows: i) isolate genome sequencing and storage of sequence information, ii) a record of isolate provenance and other phenotypic characteristics, and iii) reference tables of predefined allele sequences for the loci of interest. All of these components were implemented in the Bacterial Isolate Genome Sequence Database (BIGSDB) (Jolley and Maiden, 2010) (http://pubmlst.org/software/database/bigsdb/). BIGSDB is a scalable, open-source, web-accessible database which provides tools to store, retrieve and analyse linked phenotypic and genotypic information (Figure 4.1). The BIGSDB sequence repository may be populated with any amount of sequence data, from single gene sequences to finished genome sequences, as well as multiple contigs that make up the genome sequence of single re-sequenced isolates. When coupled with extensive information about an isolate, for example provenance data such as isolation date and location, or phenotypic characteristics such as antimicrobial susceptibility and auxotrophy, the BIGSDB provided a comprehensive toolkit for analysing the structure and function of bacteria using a population genomics approach (Jolley and Maiden, 2010).

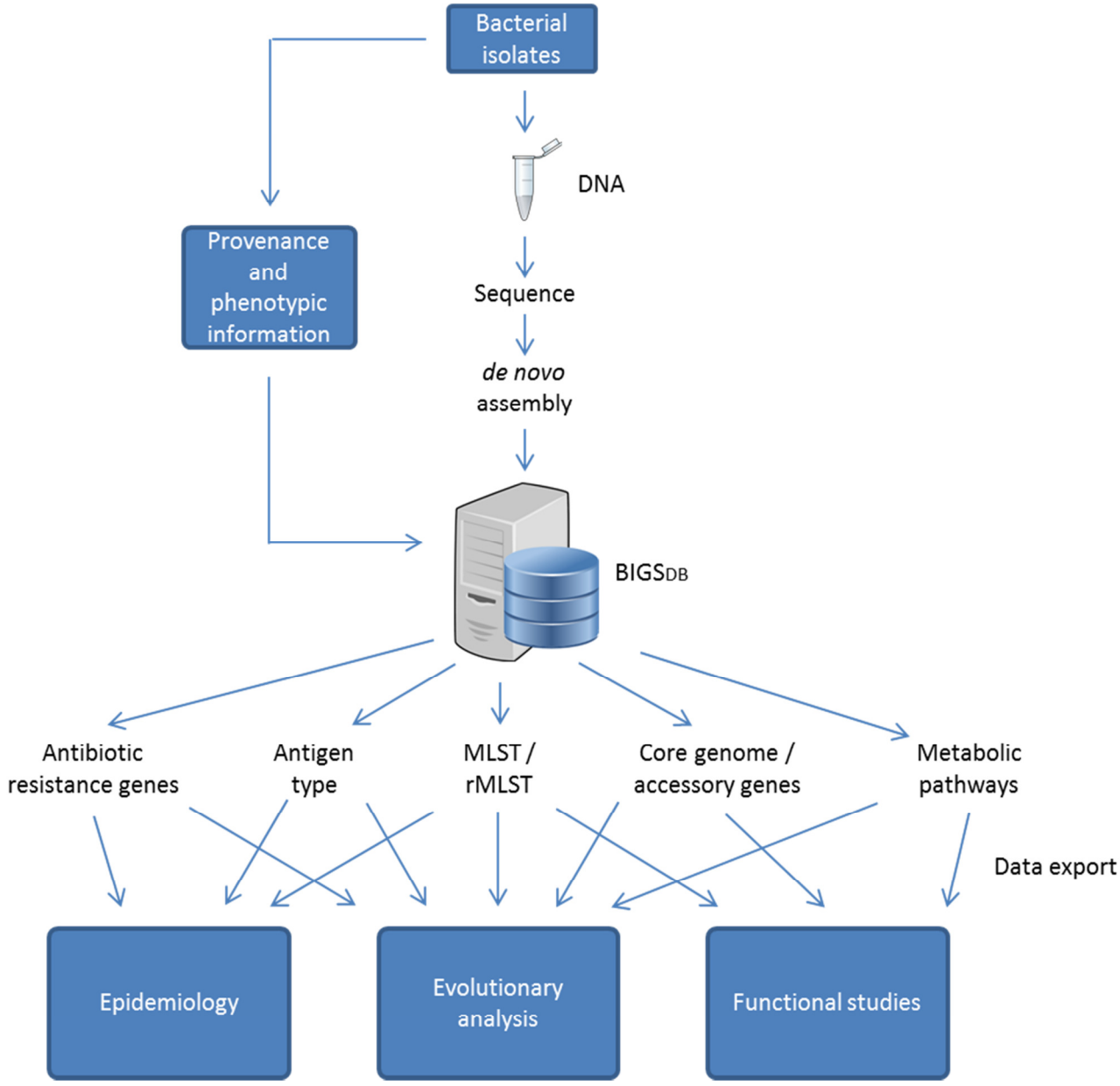


Figure 4.1: BIGSDB Structure and analysis pipelines

The BIGSDB allows users to integrate bacterial isolate provenance, phenotypic data and genotypic data from *de novo* genome sequence assemblies (adapted from Jolley and Maiden, 2010)

4.2.3. GENE-BY-GENE POPULATION GENOMICS ANALYSES USING BIGSDB

4.2.3.1. Ribosomal MLST (rMLST)

Classical MLST schemes typically analyse between six and eight housekeeping loci, providing a means of reliably identifying relationships between bacteria (Gevers et al., 2005). However, there has been concerns raised that indexing variation at less than eight loci may not provide the resolution to differentiate very closely related organisms (Achtman, 2008). At the other end of the scale, it is difficult to apply an MLST scheme based on eight loci across more distantly related bacteria, and this has meant that several different MLST schemes must be devised to cover a bacterial genus, each one unique to a particular species or closely related group of organisms. Classical MLST schemes may also not provide a practical combined taxonomic and typing approach at all levels of bacterial diversity (Jolley et al., 2012).

To address the limitations of classical MLST, BIGSDB may be used to implement ribosomal MLST (rMLST), a combined taxonomic and typing approach, and natural extension of the original seven locus MLST scheme (Jolley et al., 2012). rMLST provides resolution from the sub-species level to the whole bacterial domain, by indexing variation of sequences that encode ribosomal proteins (rps genes) (Jolley et al., 2012). Each draft genome sequence is searched for each rps locus first using BlastN at a cutoff of 70% identity over at least 50% the alignment. If no gene is identified, the stringency of the BlastN search is iteratively lowered by 5% identity to 50% identity over 50% alignment. If no locus is identified, the search is switched to TBlastX and the same iterative process undertaken. This allows diverse rps gene sequences to be identified, and each new sequence encountered is assigned a unique number, as in MLST. The final rMLST profile for each bacterial isolate comprises of a series of number defining unique sequences at genomic loci encoding 53 ribosomal proteins. rMLST has been applied in diverse systems including reclassifying *Neisseria* species (Bennett et al., 2012) and studying the epidemiology of pathogenic *Campylobacter* species (Cody et al., 2013).

4.2.3.2. "Genome-wide" MLST

When examining closely related isolates, especially those of a single species, BIGSDB also allows the user to create a "genome-wide" MLST scheme to index diversity at every locus defined within a reference sequence (Jolley and Maiden, 2010). Each locus is defined in an annotated reference sequence and iteratively searched for in the draft genome sequence of each isolate to be compared in the same way as rMLST searches for the rps genes. This allows the user to profile the genomic diversity of isolates genome-wide. Genome-wide MLST has been utilised to study the population genomics of *Campylobacter* (Sheppard et al., 2012), *Neisseria (Bennett et al., 2012)* and *Streptococcus* (Jolley and Maiden, 2010).

4.2.4. IMPLEMENTATION OF BIGSDB-BASED ANALYSES

Relationships among isolates from the *L. acidophilus* group were established using phylogenetic networks based on rMLST sequences (Section 4.2.3.1). The 53 ribosomal subunit loci identified in the automated annotation process were compared among all isolates using the BIGSDB Genome Comparator module. The distance matrix generated on the basis of shared alleles was visualized with the Neighbor-Net algorithm (Bryant and Moulton, 2004), implemented in SplitsTree version 4.8 (Huson and Bryant, 2006) within the BIGSDB Web-interface.

Isolates of *L. acidophilus* species were further analysed with the BIGSDB Genome Comparator at all loci defined in *L. acidophilus* NCFM to generate a distance matrix based on shared alleles; the matrix was visualized with Neighbor-net as above. This algorithm does not assume a tree-like structure for the data and resolves interrelationships among isolates as a phylogenetic network where appropriate. This algorithm accommodates departures from tree-like phylogeny, which for example, can result from horizontal gene transfer (Cody et al., 2013). Furthermore, the Genome Comparator was used to establish both presence/absence and count the number of allelic differences at a particular locus. At each locus, a consensus sequence was created from all alleles and the mean distance of all

alleles from the consensus sequence was calculated to provide a measure of allelic diversity per-locus. The most diverse loci were assumed to have a non-zero mean distance value. Isolates were clustered hierarchically using Ward's method based on their allelic profiles at loci with non-zero mean distances, using the heatmap2 function in the package gplots (Gregory R. Warnes et al., 2013) in R statistical software (R Development Core Team, 2012). Heatmaps were coloured using the colour palettes available in the RColorBrewer package (Neuwirth, 2011).

In order to examine sequence differences at genomic loci not defined in the *L. acidophilus* NCFM genome sequence, the *L. acidophilus* NCFM CRISPR sequence was downloaded and used to probe other *L. acidophilus* genome sequence data using BLAST+ tools implemented via the BIGSDB Web-interface (Camacho et al., 2009). Complete CRISPR sequences with 1 kb of flanking sequence on each side were downloaded and compared using the CRISPRtionary: Dictionary Creator tool, at the CRISPRdb (http://crispr.u-psud.fr/crispr/) (Grissa et al., 2007). Incomplete or partially assembled CRISPR regions were excluded from further analysis.

4.2.5. EXPOSURE OF *L. ACIDOPHILUS* NCFM TO METHYL METHANE SULPHONATE (MMS)

Mutagenesis with MMS was performed in two stages, the first to estimate an MMS exposure that would ensure a 99% reduction in viability of a culture of *L. acidophilus* NCFM, and the second to mutagenise *L. acidophilus* NCFM with three sequential exposures to MMS, retaining two isolates for genome resequencing at each culture passage stage as follows. Overnight cultures of *L. acidophilus* NCFM were grown at 37℃ in MRS broth. Input bacterial suspensions were enumerated using a viable drop count method. Serial dilutions were performed in MRS broth; 10 µl drops of bacterial suspension were aspirated onto the surface of an MRS agar plate in triplicate and incubated at 37℃ for 24 hours. Individual colonies were counted and the number of viable cells estimated.

MMS was added to 1 ml of suspension to a final concentration of 3, 4 or 5% (v/v) for exposure times of 10, 15 and 30 minutes. After exposure, suspensions were separated with centrifugation and the supernatant containing MMS aspirated. 1 ml of fresh MRS broth was added to the pellet to re-suspend, and the drop count enumeration procedure was repeated to establish the output from the test. A percentage reduction in culture viability was calculated using the ratio of output enumeration to input enumeration.

Subsequently, *L. acidophilus* NCFM cultures were subjected to three rounds of mutagenesis by exposure to MMS at 5 % (v/v) for 15 minutes, an exposure which resulted in 99% loss of viability. As previously, 1 ml of bacterial overnight culture was enumerated, exposed to MMS, enumerated again to confirm the 99% reduction in viability and then frozen at -80℃ after the addition of 8% DMSO as a cryoprotectant. This freezer stock was revived onto MRS agar and fresh overnight bacterial cultures were inoculated from three colonies. High molecular weight DNA was prepared from two overnight bacterial cultures as described previously (Section 4.2.1.2) and the third was subjected to the same MMS mutagenesis exposure. This was repeated for a third round, ensuring DNA was prepared from six MMS-exposed single colony-based isolate cultures. High molecular weight DNA from these isolates was subject to the same sequencing and assembly procedures described in Section 4.2.1.2, RAPD PCR as described in Chapter 2, and assembled data were deposited in the PubMLST database before further analysis. The Genome Comparator tool implemented in the BIGSDB was used to identify loci that had different alleles to the reference *L. acidophilus* NCFM genome. These were assumed to be mutations induced by exposure to MMS.

4.3. RESULTS

The results are divided into three subsections as follows. The first describes a small scale *L. acidophilus* genomic diversity survey, mapping the genomes of three *L. acidophilus* isolates; CUL21 and CUL60 from the Lab4® capsule and *L. acidophilus* LMG 11428, a culture collection isolate with the earliest-recorded deposition date (1922, Table 4.2). The second section describes a large-scale genomic diversity survey of *L. acidophilus* isolates. Overall, 30 *L. acidophilus* genome sequences were included in this broader study, reflecting the progressive shift toward attaining highly-economical (virtually) complete prokaryotic genome sequences. Finally, the third results section sought to establish why so little genomic variation was observed between *L. acidophilus* isolates, particularly considering their reported ecological niche, the GIT, is a known mutagenic environment.

4.3.1. SMALL SCALE *L. ACIDOPHILUS* EVOLUTIONARY GENOMICS

A visual comparison of the genome sequence of *L. acidophilus* NCFM with *L. acidophilus* CUL21 and CUL60, showed synteny across the entirety of their genome sequences (Figure 4.2). Single Nucleotide Polymorphism (SNP) detection identified a total of 87 polymorphisms between *L. acidophilus* CUL21 and *L. acidophilus* NCFM, of which 13 were non-coding, 22 encoded synonymous mutations and 48 encoded non-synonymous mutations. Between *L. acidophilus* NCFM and *L. acidophilus* CUL60, a total of 85 SNPs were observed, 15 non-coding, 21 encoding synonymous mutations and 49 encoding non-synonymous mutations. Of 364 SNPs between *L. acidophilus* LMG11428 and *L. acidophilus* NCFM, 48 were non-coding, 57 encoded synonymous mutations and the remaining 258 encoded none non-synonymous mutations. When SNP locations were compared for all strains, 76 SNPs were found to have identical locations and with same alternate base call at each of these positions for CUL21, CUL60 and LMG 11428.

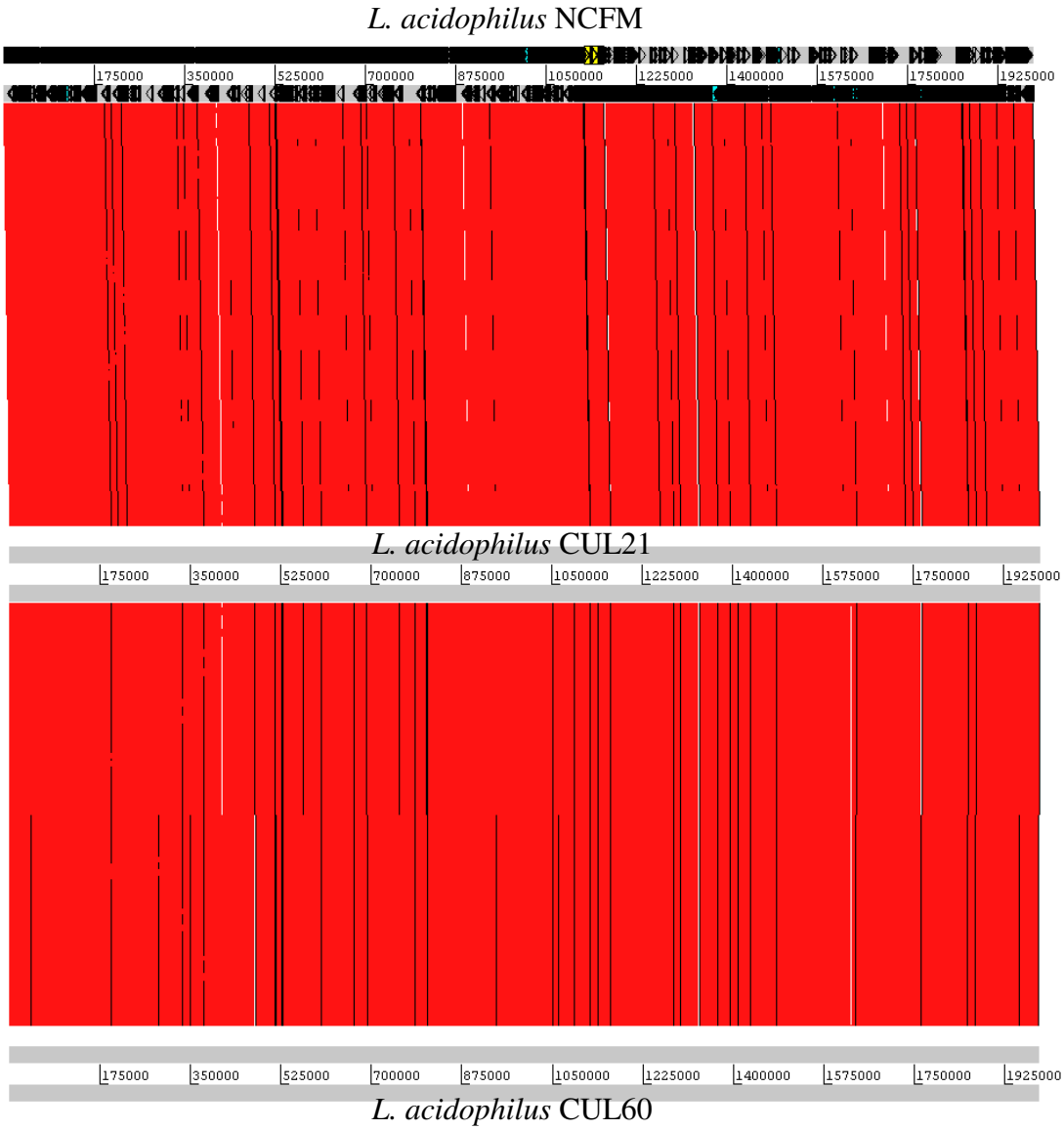


Figure 4.2: Whole genome comparison of *L. acidophilus* NCFM to *L. acidophilus* CUL21 and *L. acidophilus* CUL60.

Genome sequences are represented by horizontal lines with *L. acidophilus* NCFM as the top-most horizontal lines. Regions of similar sequence are linked with lines between genomes. Sequence identity is indicated by shade intensity. Red indicates similar sequence in the forward direction. The scale bar indicates size in bases across the 2 Mb genome comparison.

To establish the phylogenetic relationships between these isolates, whole-genome consensus sequences were created and aligned. A series of whole genome phylogenies can be seen in Figure 4.3. Examining phylogenetic relationships at three different taxonomic resolutions, the first (Figure 4.3; panel A) shows no phylogenetic distance between *L. acidophilus* isolates when they were compared to reference genomes for 5 species within the *L. acidophilus* group. The second (Figure 4.3; panel B) included just *L. amylovorus* GRL1112, the closest relative of *L. acidophilus*, based on 16S rRNA gene phylogeny (Figure 1.2), and again did not show any phylogenetic distance between the *L. acidophilus* isolates. When just *L. acidophilus* isolates were examined, phylogenetic distance was observed (Figure 4.3; panel C). The distances were objectively small, considering the low numbers of SNPs detected (maximum 364 SNPs over 2 Mb genome), but it can clearly be seen that *L. acidophilus* CUL21 and CUL60 are more closely related to one another than *L. acidophilus* NCFM, and that these 3 isolates are more distant to the oldest deposited strain, *L. acidophilus* LMG 11428.

The overall genomic similarity of these isolates presented a considerable challenge when examining their genomic diversity, as any variation was hidden within the overwhelming identity of their genome sequence. Therefore, for the subsequent large scale *L. acidophilus* genomic diversity survey, the comparative strategy used to establish the evolutionary results of polymorphic regions was adapted to consider single genes as the unit of comparison, rather than the whole genome sequence.

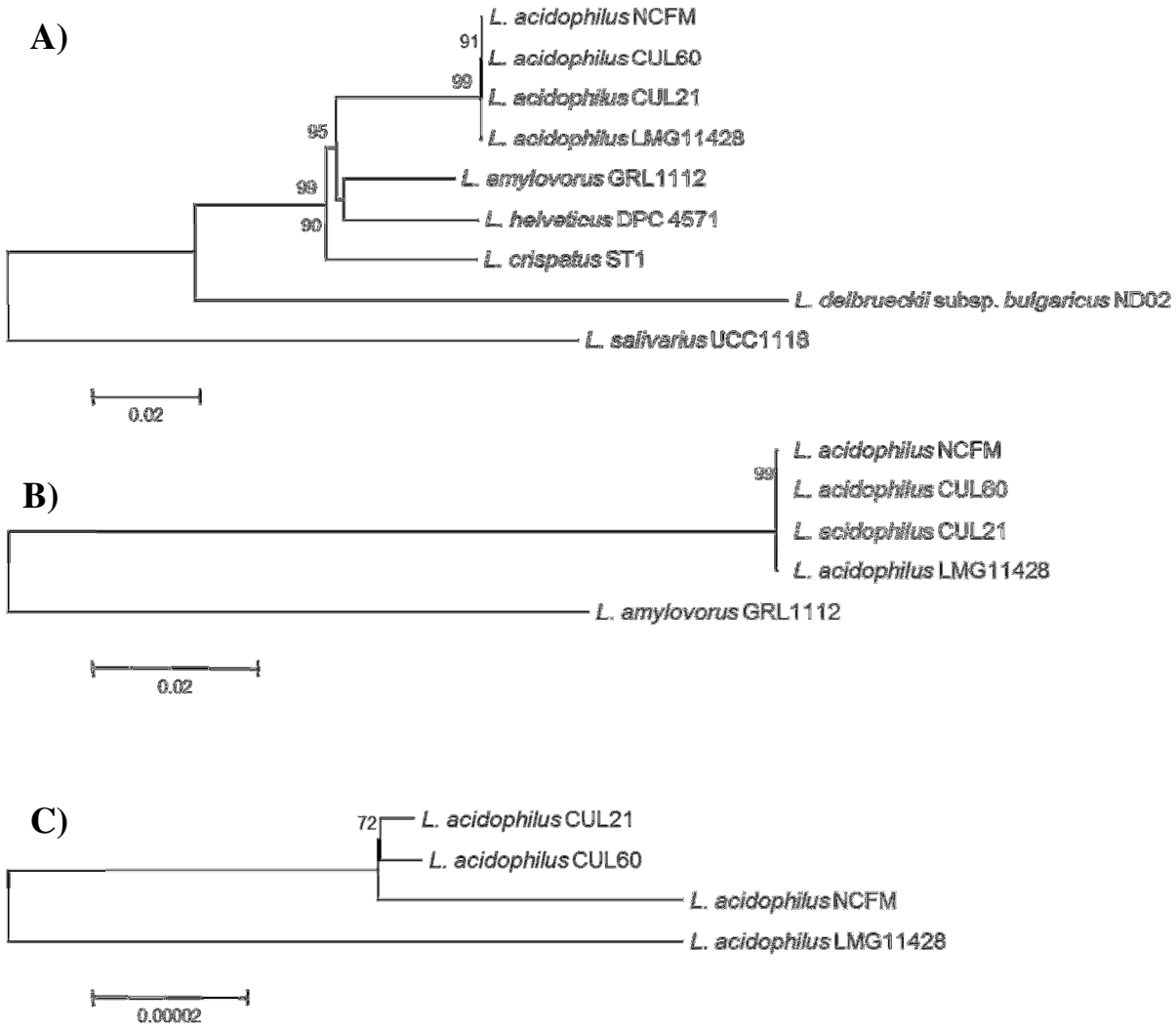


Figure 4.3: Phylogenetic trees of lactobacilli closely related to *L. acidophilus*

Phylogenetic analysis of aligned whole genome sequences at three levels of resolution. The *L. acidophilus* group (panel A), *L. acidophilus* and *L. amylovorus* (panel B), and just *L. acidophilus* (panel C). The genetic distance scale and bootstrap values (500 replicates) are indicated.

4.3.2. EXPANDED *L. ACIDOPHILUS* GENOMIC DIVERSITY STUDY

4.3.2.1. Assessing genomic diversity of the *L. acidophilus* group using rMLST

To contextualise the diversity of *L. acidophilus* isolates, rMLST was performed on isolates from the *L. acidophilus* group. 53 genes that encode ribosomal proteins were identified in all genome sequences (25 *L. acidophilus* isolates represented by 30 genome sequences, and 19 other *L. acidophilus* group genome sequences). The *rps* genes represent 20,640 nucleotides in *L. acidophilus* NCFM and hence account for any observed variation in just over 1% of its total genome sequence.

A Neighbour Joining phylogenetic tree of the concatenated sequences of all rMLST loci was able to resolve each species within the *L. acidophilus* group (Figure 4.4), despite some species sharing 99% of their 16S rRNA gene sequences (Bull et al., 2012). The phylogeny illustrates the strain level diversity of the *L. acidophilus* group, with *L. gasseri, L. johnsonii*, *L. delbruekii* and *L. helveticus* isolates diverging earlier than isolates of *L. jensenii* and *L. acidophilus*. *L. acidophilus* isolates fall as a single group, diverging early from other *L. acidophilus* group species, and also diverging recently among themselves. Further analysis of isolates of *L. acidophilus* was conducted using DNA sequence from all protein coding regions to improve resolution.

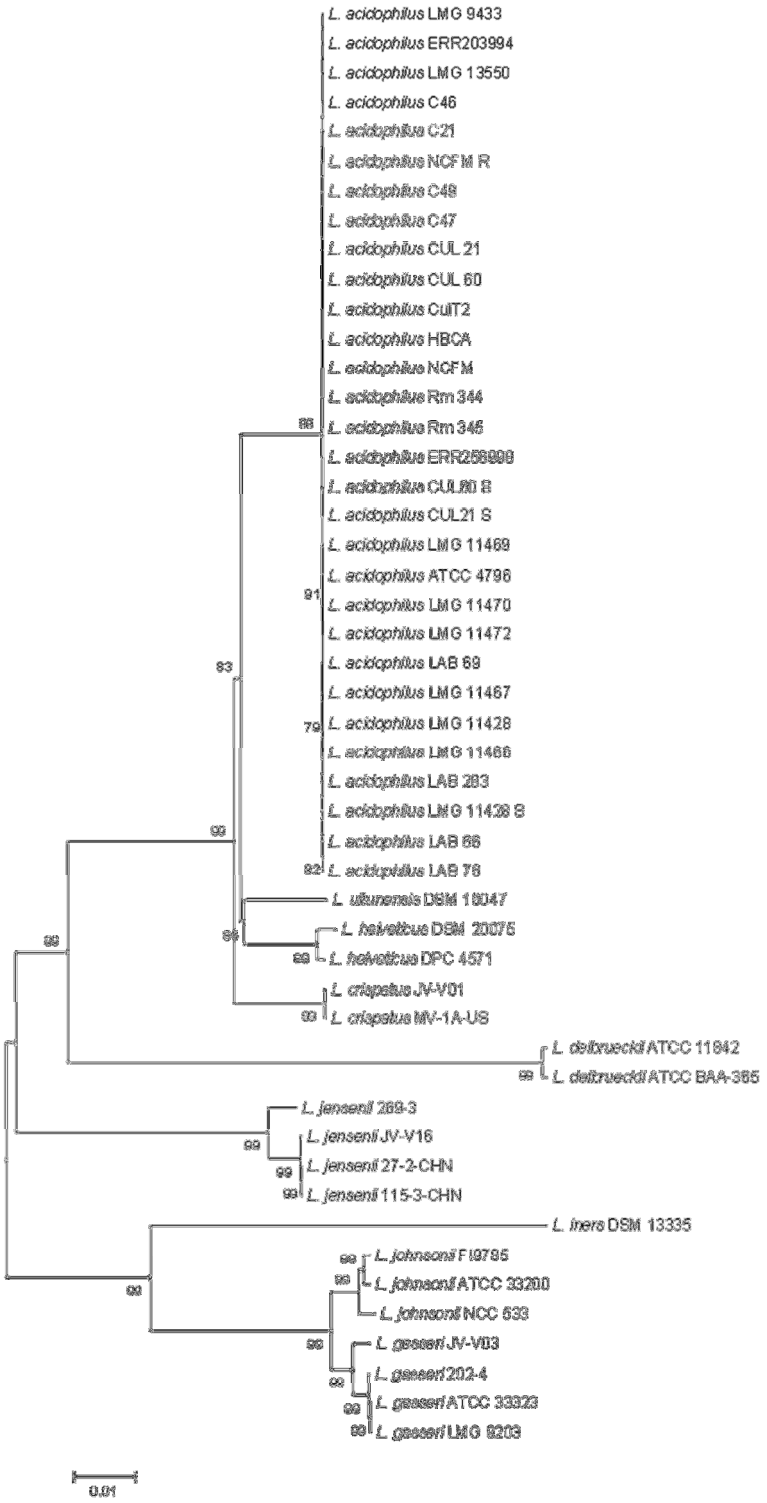


Figure 4.4: Neighbour Joining phylogeny of concatenated ribosomal protein genes from *L. acidophilus* group isolates

The tree was based on the concatenated sequences of 53 *rps* loci (20, 640 nucleotides). A bootstrap test of reliability was performed for 500 replications.

4.3.2.2. Genomic diversity of *L. acidophilus* isolates

4.3.2.2.1. ASSESSING GENOMIC DIVERSITY OF *L. ACIDOPHILUS* USING A GENOME-WIDE GENE-BY-GENE APPROACH

A Neighbour-Net analysis of the concatenated sequences of all shared loci encoded in the *L. acidophilus* NCFM genome sequence was able to resolve each strain within *L. acidophilus* (Figure 4.5). Isolates that had been deposited in duplicate locations in the same culture collection, such as *L. acidophilus* LMG 9433 and *L. acidophilus* LMG 13550, showed demonstrably similar protein coding gene sequences across their genomes. The same isolate sequenced as part of different studies using similar sequencing technologies, such as *L. acidophilus* LMG 9433, represented by sequences LMG 9433 and ERR203994, also show co-localising placement in the Neighbour-Net. The same isolate sequenced as a part of different studies using different sequencing and assembly technologies with the two genome sequences generated from isolates acquired from different culture collections also show close placement on the Neighbour-Net; *L. acidophilus* LMG 11470 (Illumina HiSeq2000, Velvet, this study) and ATCC 4796 (454-GS-FLX, Newbler, Human Microbiome Project) (Figure 4.5, Table 4.2). The *L. acidophilus* NCFM reference genome sequence was generated in 2005 using Sanger sequencing (Altermann et al., 2005) and its sequence was observed to be markedly different to the isolate of *L. acidophilus* NCFM resequenced as part of this study (Figure 4.5).

It was also immediately obvious that genome sequences generated from "commercial" *L. acidophilus* isolates, from Cultech or isolated directly from probiotic products formed a tight cluster in the Neighbour-Net (Figure 4.5). In comparison, the genome sequences generated from isolates from culture collections were more diffuse in their placement in the Neighbour-Net (Figure 4.5). It should also be noted that the genome-wide protein-coding sequences of all commercial isolates were more similar to one another than repeated testing of the same isolate from different studies and sequencing technologies. For example all commercial isolates formed a tighter cluster than the cluster created by *L. acidophilus* LMG 9433, LMG 13550 and ERR203994 (Figure 4.5).

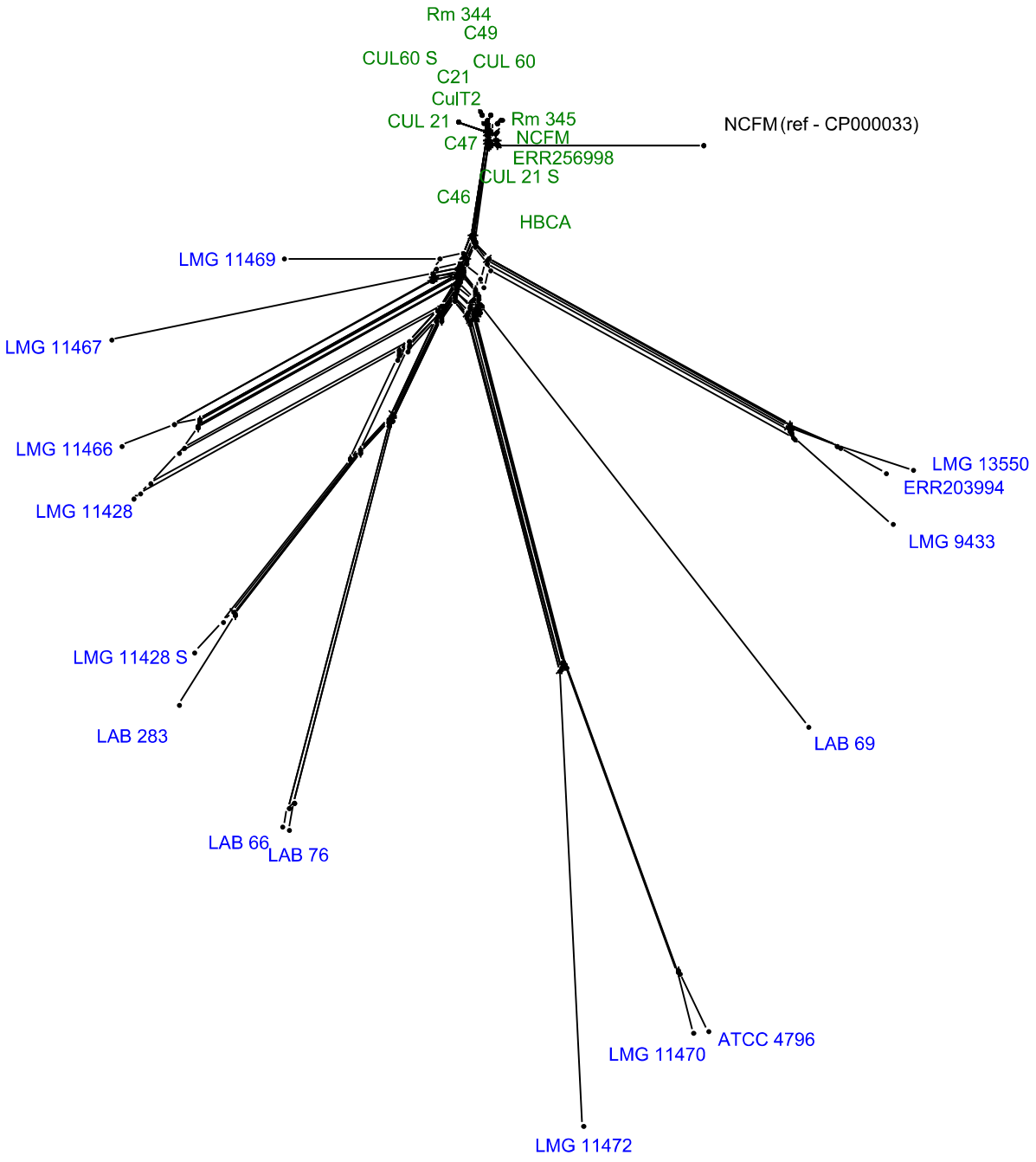


Figure 4.5: Neighbour-Net of concatenated sequences of all protein coding genes from *L. acidophilus* isolates.

The analysis was based on the concatenated sequences of 1864 predicted protein coding loci. An overall fit of 99.49 % indicated that this network accurately represented the original sequence data. Isolates are coloured to represent their history with the blue isolates derived from culture collections and the green isolates representing commercial isolates from Cultech Ltd. or probiotic products (see Table 4.2 for isolate details).

4.3.2.2.2. REGIONS NOT PRESENT IN *L. ACIDOPHILUS* ISOLATES

All *L. acidophilus* isolates shared a markedly high (1828/1862) number of protein coding DNA sequences with *L. acidophilus* NCFM reference to which they were compared (Figure 4.6). The remaining loci that were variably present in *L. acidophilus* isolates are shown in Figure 4.7. Interestingly, the majority of these fluctuating loci were attributed to the three PAU regions of *L. acidophilus* NCFM (see Section 1.3.3) or loci closely associated with these regions. An additional variant region was composed of three consecutive gene loci, LBA0058, LBA0059 and LBA0060, also encoded within phage-related DNA, but distinct from the PAU DNA. When isolates of *L. acidophilus* were separated based on their history (commercial and culture collection), differences in the distribution of the three PAU regions was evident. The PAU1 locus was widely distributed across all isolates, culture collection and commercial, with the exception of CUL21 and C47. Both of these isolates are from Cultech, with CUL21 used as part of the Lab4® probiotic. Other commercial isolates had the other PAU regions, 2 and 3, intact and shared more loci found in *L. acidophilus* NCFM than isolates from culture collections (Figure 4.7). Culture collection isolates demonstrated more variably present all loci, including PAU2 and PAU3, and other coding regions (Figure 4.7).

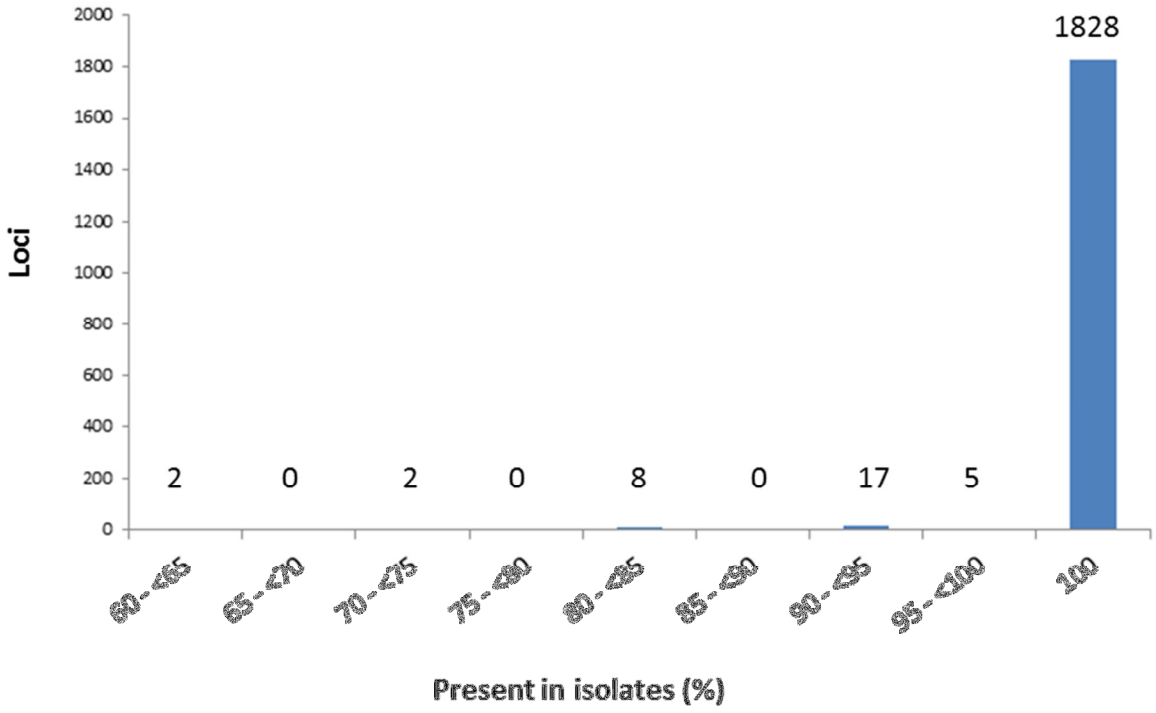
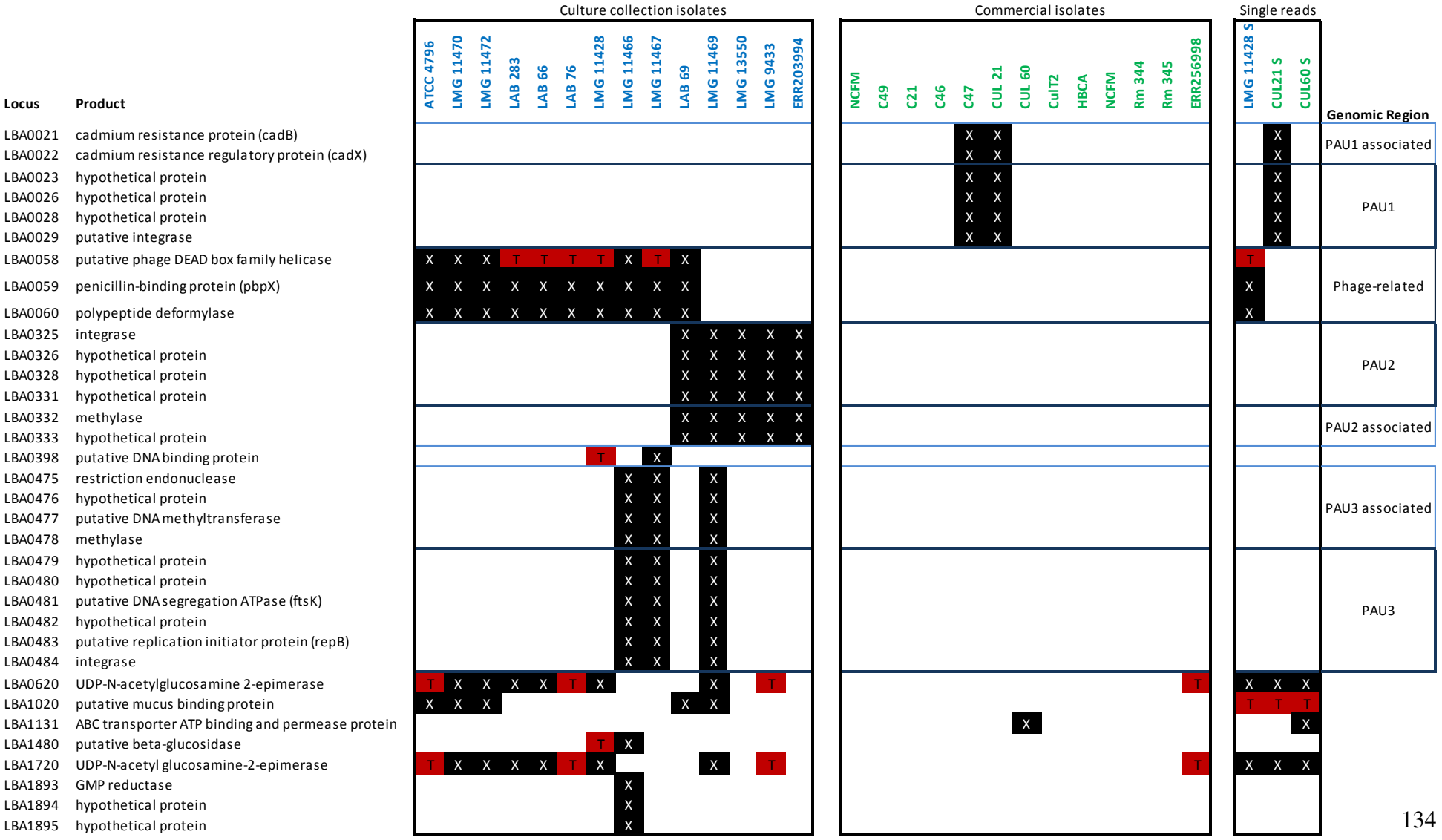


Figure 4.6: *L. acidophilus* NCFM protein coding loci found in other *L. acidophilus* isolates.

The distribution of all protein-coding loci defined in the *L. acidophilus* NCFM genome sequence, in all other *L. acidophilus* isolates is represented. 1828 loci are found in all isolates, 17 loci are found in between 90% and 95% of isolates etc.


Culture collection isolates
Commercial isolates
Single reads
ATCC 4796
LMG 11470
LMG 11472
LAB 283
LAB 66
LAB 76
LMG 11428
LMG 11466
LMG 11467
LAB 69
LMG 11469
LMG 13550
LMG 9433
ERR203994
NCFM
C49
C21
C46
C47
CUL 21
CUL 60
CulT2
HBCA
NCFM
Rm 344
Rm 345
ERR256998
LMG 11428 S
CUL21 S
CUL60 S
Locus
Product
Genomic Region
LBA0021 cadmium resistance protein (cadB)
LBA0022 cadmium resistance regulatory protein (cadX)
PAU1 associated
LBA0023 hypothetical protein
LBA0026 hypothetical protein
LBA0028 hypothetical protein
LBA0029 putative integrase
PAU1
LBA0058 putative phage DEAD box family helicase
LBA0059 penicillin-binding protein (pbpX)
LBA0060 polypeptide deformylase
Phage-related
LBA0325 integrase
LBA0326 hypothetical protein
LBA0328 hypothetical protein
LBA0331 hypothetical protein
PAU2
LBA0332 methylase
LBA0333 hypothetical protein
PAU2 associated
LBA0398 putative DNA binding protein
LBA0475 restriction endonuclease
LBA0476 hypothetical protein
LBA0477 putative DNA methyltransferase
LBA0478 methylase
PAU3 associated
LBA0479 hypothetical protein
LBA0480 hypothetical protein
LBA0481 putative DNA segregation ATPase (ftsK)
LBA0482 hypothetical protein
LBA0483 putative replication initiator protein (repB)
LBA0484 integrase
PAU3
LBA0620 UDP-N-acetylglucosamine 2-epimerase
LBA1020 putative mucus binding protein
LBA1131 ABC transporter ATP binding and permease protein
LBA1480 putative beta-glucosidase
LBA1720 UDP-N-acetyl glucosamine-2-epimerase
LBA1893 GMP reductase
LBA1894 hypothetical protein
LBA1895 hypothetical protein

← ***Previous Page***

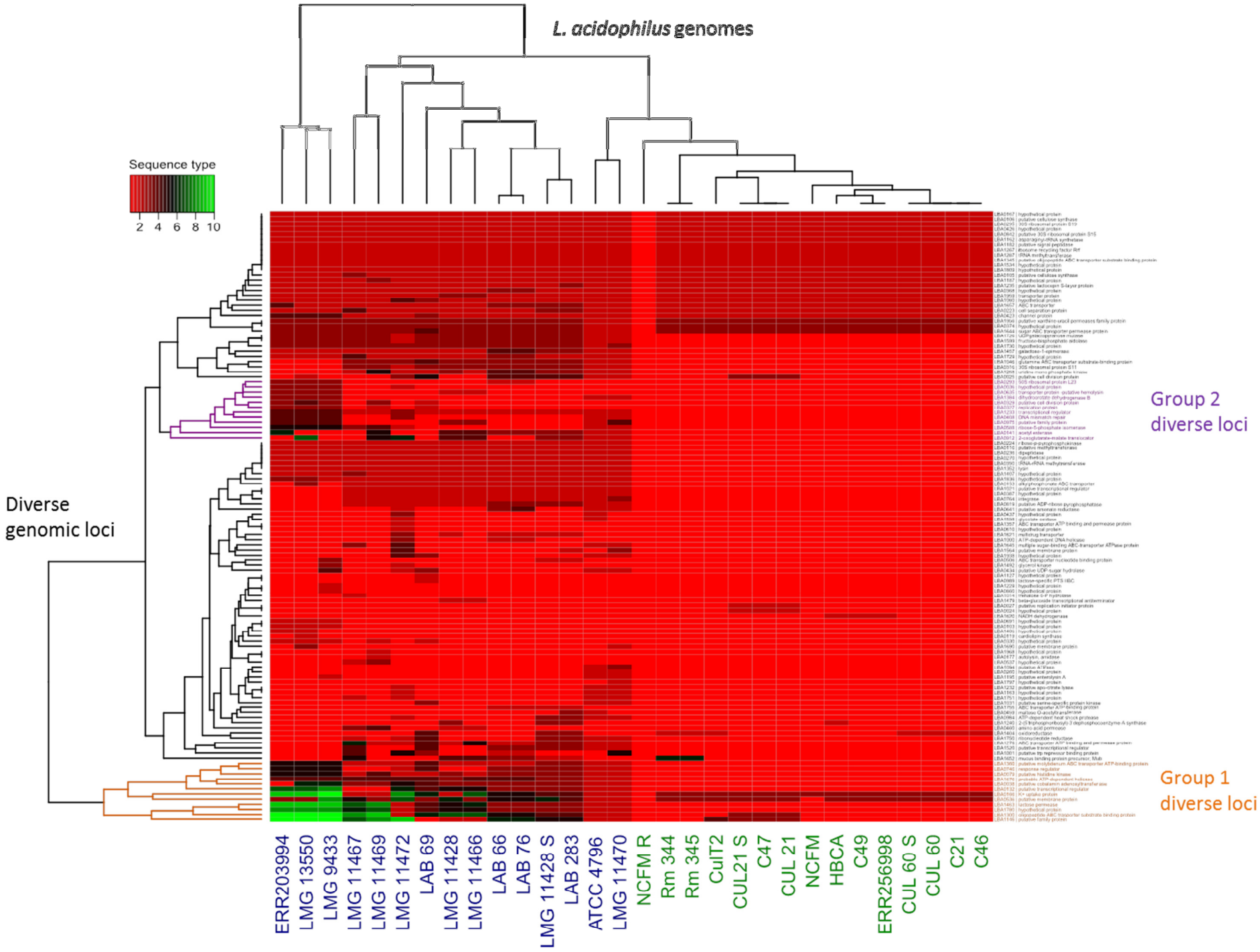
Figure 4.7: Variably present protein coding loci

Loci are defined in the *L. acidophilus* NCFM genome sequence (GenBank: CP000033), and representative predicted gene products from the same sequence are listed. Completely missing loci are noted with an X, and truncated loci, indication that a proportion of the locus present at the end of a sequence contig, are noted with a T. The three PAU regions and phage related DNA within of the *L. acidophilus* NCFM genome are marked (add the genomic region header and phage-related DNA label), and isolate names are coloured blue for culture collection isolates and green for commercial isolates. Also included in a separate column is the analysis of the genome sequences generated from the small scale genome resequencing study (section 4.3.1)

4.3.2.2.3. LOCI SHOWING SEQUENCE DIVERSITY

Sequence diversity was estimated by calculating mean distances for all protein coding loci defined in the *L. acidophilus* NCFM reference genome. A consensus sequence was generated from the sequences of all isolates at each locus, and then the distance of each individual isolate sequence from this consensus derived for each locus. The mean of these distances provided a simple way of assessing diversity at each protein coding locus. A mean distance of zero was observed at over 70% of genomic loci (1305/1864). The remaining loci that showed non-zero mean distances were filtered to remove: i) truncated loci present at the ends of sequence contigs, and ii) paralogous loci that were mostly attributed to multicopy transposases. The remaining loci were then compared across all isolates, and each time a new sequence was encountered, this was assigned a new "allele" number. An "allelic profile" for each isolate was then generated, similar to a traditional MLST scheme, but encompassing only the diverse protein coding loci. Ten different alleles were encountered at the most diverse locus and this variation in correlation with the genome sequences was used to generate a heatmap of locus diversity (Figure 4.8). When isolates were clustered based on the similarity of their allelic profiles, culture collection isolates again clearly separated from commercial isolates (Figure 4.8). Commercial isolates were much more homogenous in their allelic profiles, and more similar in sequence to *L. acidophilus* NCFM. The culture collection isolates were much more diverse, although

duplicate isolates from different culture collections such as *L. acidophilus* ATCC 4796 and LMG 11470, and *L. acidophilus* LMG 9433, LMG 13550 and ERR203994, clustered together respectively.


L. acidophilus genomes
Sequence type
2 4 6 8 10
Diverse genomic loci
Group 2 diverse loci
Group 1 diverse loci
ERR203994
LMG 13550
LMG 9433
LMG 11467
LMG 11469
LMG 11472
LAB 69
LMG 11428
LMG 11466
LAB 66
LAB 76
LMG 11428 S
LAB 283
ATCC 4796
LMG 11470
NCFM R
Rm 344
Rm 345
CulT2
CUL21 S
C47
CUL 21
NCFM
HBCA
C49
ERR256998
CUL 60 S
CUL 60
C21
C46

← *Previous page*

Figure 4.8: Variable loci in *L. acidophilus* genome sequences

L. acidophilus genome sequences (columns) are represented by their most diverse loci (rows), forming an allelic profile for each isolate. Each locus in each genome sequence is coloured from red to green relative to its allele number (1 is red, 10 is green). As new allele numbers are assigned only when a new sequence is encountered at that particular locus, the most diverse alleles have a larger amount of green cells. The genome sequences are clustered according to their allelic profile similarity, and the loci according to their overall diversity. Again, genome sequences are coloured based on their history, culture collection isolates in blue and commercial isolates in green. Two clusters of diverse loci examined in further detail are highlighted in purple (Group 2) and orange (Group 1).

Two groups of diverse loci, group 1 and group 2, were identified in heatmap analysis (Figure 1.8) and further details on these coding sequences is provided in Figure 4.9. The group 1 loci (Figure 1.9; orange font) were highly diverse in isolates from culture collections, yet distinctly homogenous in commercial isolates. Loci encoding outer-membrane and transport-related functions were most diverse coding sequences in these group 1 loci (LBA1300, LBA0166 and LBA1463). LBA1146 displayed a similar level of diversity but has no annotated function attributed, although it has close (>80% sequence identity) homologues in *Lactobacillus kefiranofaciens* ZW3 and *Lactobacillus crispatus* ST1. LBA1360 and LBA0079 were less diverse and also associated with cross-membrane transport. Although up to ten different alleles were encountered at these loci, overall mean distances were below 0.008 and generally in the range of 0.001 to 0.002 (Figure 1.9).

The second group of diverse loci, group 2, (Figure 1.9, purple font), were entirely homogenous in sequence in the commercial isolates, and diverse in the culture collection isolates, but less so than the group 1 sequences. Two loci had large mean distances (LBA0327 and LBA0329, 0.043 and 0.038 respectively), but this may be attributed to them being part of PAU2, of which parts were observed to be variably present in *L. acidophilus* isolates (Section 4.3.2.2.2). Of the remaining loci within group 2, three

(LBA0293, LBA0408 and LBA1384) were associated with "housekeeping" type products such as ribosomal proteins and DNA repair.

	Locus	Product	SEED Functional Category	ERR203994	LMG 13550	LMG 9433	LMG 11467	LMG 11469	LMG 11472	LAB 69	LMG 11428	LMG 11466	LAB 66	LAB 76	LMG 11428 S	LAB 283	ATCC 4796	LMG 11470	NCFM R	Rm 344	Rm 345	CulT2	CUL 21 S	C47	CUL 21	NCFM	HBCA	C49	ERR256998	CUL 60 S	CUL 60	C21	C46	Mean Distance
Group 1 diverse loci	LBA1300	oligopeptide ABC trasporter substrate binding protein	ABC transporter	10	10	10	8	9	2	6	7	7	5	5	4	4	2	2	1	1	1	1	3	3	3	1	1	1	1	1	1	1	0.008
	LBA1360	ABC transporter ATP-binding protein	none	5	5	5	2	2	2	4	2	2	2	2	3	3	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.001
	LBA0746	response regulator	none	5	5	5	1	1	2	4	1	1	3	3	3	3	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.002
	LBA0079	putative histidine kinase	Heme uptake and utilisation	5	5	5	2	4	2	2	3	2	3	3	3	3	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.001
	LBA1676	probable ATP-dependent helicase	none	4	4	4	4	4	4	4	3	4	4	4	3	3	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.001
	LBA0038	putative cobalamin adenosyltransferase	none	1	6	6	3	3	5	4	3	3	3	3	3	3	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.001
	LBA0132	putative transcriptional regulator	none	7	7	8	1	1	6	4	5	5	3	3	3	3	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.001
	LBA0166	K+ uptake protein	Potassium homeostasis	9	9	10	2	2	8	6	2	7	5	5	4	4	2	2	1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	0.001
	LBA0536	putative membrane protein	none	3	3	8	5	3	7	3	6	3	5	5	6	4	2	2	1	3	3	3	3	3	3	1	3	3	3	3	3	3	3	0.002
	LBA1463	lactose permease	Lactose utilization	9	9	9	7	8	2	4	5	6	3	3	3	3	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.001
	LBA1780	hypothetical protein	N/A	8	8	8	6	7	2	4	5	5	3	3	3	3	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.001
	LBA1146	putative family protein	none	10	10	10	7	8	9	1	2	2	6	6	5	5	2	2	1	1	1	4	3	3	3	1	1	1	1	1	1	1	1	0.002
Group 2 diverse loci	LBA0036	hypothetical protein	N/A	3	3	3	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.001
	LBA0141	acetyl esterase	none	6	1	1	1	5	1	1	4	4	3	3	3	3	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.002
	LBA0293	50S ribosomal protein L23	Ribosome	3	3	3	1	1	1	1	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.001
	LBA0327	replication protein	none	3	3	3	1	2	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.043
	LBA0329	putative cell division protein	none	3	4	3	1	3	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.038
	LBA0408	DNA mismatch repair	DNA repair, bacterial MutL-MutS system	4	4	4	1	1	3	1	1	1	1	1	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.001
	LBA0588	ribose-5-phosphate isomerase	D-ribose utilization, pentose phosphate pathway	5	5	5	3	4	2	1	1	1	1	1	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.002
	LBA0635	transporter protein -putative hemolysin	none	3	3	3	1	1	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.001
	LBA0912	2-oxoglutarate-malate translocator	none	1	7	1	1	5	6	1	4	4	1	1	3	3	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.002
	LBA0975	putative family protein	none	4	4	4	1	1	1	1	3	3	1	1	1	1	2	4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.001
	LBA1233	transcriptional regulator	none	4	4	4	2	2	3	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.001
	LBA1384	dihydroorotate dehydrogenase B, catalytic unit	*de novo* Pyrimidine synthesis	3	3	3	1	1	1	1	1	1	1	1	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.001

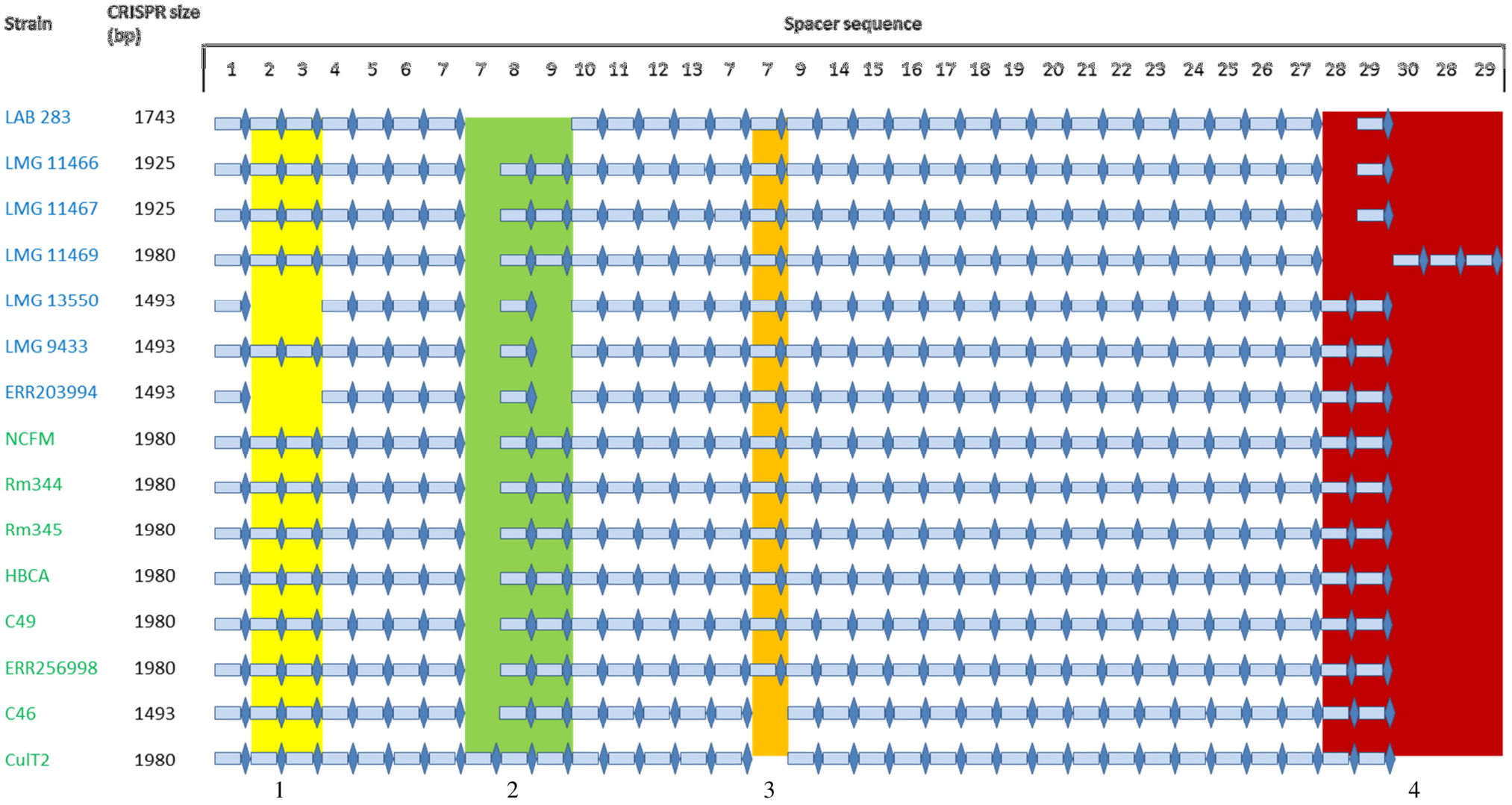
←Previous page

Figure 4.9: Selected diverse loci in *L. acidophilus*

Two groups of selected diverse loci in *L. acidophilus*, group 1 and group 2, are expanded from Figure 4.8. Loci are coloured as per Figure 4.8 and their SEED functional categories listed. The allelic profile of each isolate at each of these loci is coloured in the same way as Figure 4.8. The overall mean distance of each locus is noted. (again add sub-header labels to group 1 and 2).

4.3.3. *L. ACIDOPHILUS* CRISPR REGION DIVERSITY

All complete (on a single sequence contig) CRISPR regions from *L. acidophilus* isolates were extracted and analysed (Figure 4.10). The archetypal *L. acidophilus* CRISPR sequence is defined in the genome sequence of *L. acidophilus* NCFM, and consists of 33 units of a repeat region and a spacer region. The *L. acidophilus* NCFM CRISPR had the full gamut of spacer sequences and comprised the longest sequence length of 1980 bp. The shortest CRISPR sequences were 1493 bp in length, and missing up to four spacers (Figure 4.10; *L. acidophilus* LMG 13550, LMG 9433, ERR203994 and C46). Four regions of diversity in *L. acidophilus* CRISPR sequences were identified. BlastN was used to find similar sequences to these polymorphic spacers (Table 4.3). Unexpectedly, seven out of eight of thee polymorphic spacers showed at least partial similarity to *L. acidophilus* ribosomal protein encoding genes (Table 4.3).


Strain
CRISPR size (bp)
Spacer sequence
1 2 3 4 5 6 7 7 8 9 10 11 12 13 7 7 9 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 28 29
LAB 283 1743
LMG 11466 1925
LMG 11467 1925
LMG 11469 1980
LMG 13550 1493
LMG 9433 1493
ERR203994 1493
NCFM 1980
Rm344 1980
Rm345 1980
HBCA 1980
C49 1980
ERR256998 1980
C46 1493
CulT2 1980
1
2
3
4

←Previous page

Figure 4.10: *L. acidophilus* CRISPR sequences

The complete CRISPR region from each *L. acidophilus* isolate is represented by a series of repeats (dark blue diamond) and unique spacers (light blue oblong). Each unique spacer sequence is assigned a new number, which can be seen above its relevant spacer oblong. Four regions of polymorphism are highlighted with coloured boxes, and numbered below. The size of each CRISPR region is given in bp.

Table 4.3: Sequences similar to polymorphic CRISPR spacers

Diversity region	Spacer No.	Spacer sequence	Similar sequences	Sequence coverage (%)	Identity (%)
1	2	TGGAATCTCATCGTAAGAAATAAGTCGCATATA	*L. acidophilus* 16S rRNA gene (partial)	45	100
	3	CCTTTTCCTAGGATCTTCATAAGCTTCTCGCCA	*L. acidophilus* 23S rRNA gene (partial)	39	92
2	7	CGGCAATTTTTGAAACAAACAACTATGTATATA	*L. acidophilus* 23S rRNA gene (partial)	54	100
	8	AAATAAGGAAGATATTGCCACCCTCGGTACCCA	*L. acidophilus* 23S / 16S rRNA genes (partial)	30	100
	9	ACAAGTTTTGCTCTAACCATGATGTTGTAAACA	*L. acidophilus* 23S rRNA gene (partial)	45	100
3	7	CGGCAATTTTTGAAACAAACAACTATGTATATA	*L. acidophilus* 23S rRNA gene (partial)	54	100
4	28	TAATACGTAGGTGGCAAGCGTTGTCCGGATTTA	16S rRNA gene conserved region	100	100
	29	ATAAAAATAAGAGGAAACCACCGTTTTCTCTTA	*L. acidophilus* 23S rRNA gene (partial)	27	100
	30	TTTTGGGCGTTAATCCCGTGGCGAATTAATTCG			

4.3.4. MUTAGENESIS OF *L. ACIDOPHILUS* NCFM WITH MMS

To replicate and exceed the mutagenic capacity of the GIT as the natural niche of *L. acidophilus*, strain NCFM was exposed to MMS, which causes DNA alkylation and double-stranded DNA breaks. The range of exposure strategies was chosen based on successful previous mutagenesis of *L. delbrueckii* (Demirci and Pometto, 1992). *L acidophilus* NCFM was exposed to MMS at final media concentrations of ranging from 3% to 5% (v/v) for durations of 10, 15 and 30 minutes. An exposure of 15 minutes to 5%(v/v) MMS was selected as the exposure required to reduce viability of the bacterial culture by 99% (Figure 4.11). Three exposures were performed at this MMS exposure condition and duplicate isolates collected after each exposure and their genomic DNA re-sequenced (Table 4.4). No gross differences in genome structure between the MMS exposed isolates and the parent *L. acidophilus* NCFM isolate were observed using RAPD profile comparisons (Figure 4.12).

Draft genomes were assembled for each isolate, and their sequences compared at all protein coding loci defined in the *L. acidophilus* NCFM genome sequence, as in Section 4.3.2. Of 1862 protein coding loci, 1732 were identical in all isolates. The remaining polymorphic loci were screened for paralogous and truncated loci, and after their removal 38 polymorphic loci were available to elucidate the results of MMS exposure (Table 4.5). None of these loci differed to the *L. acidophilus* NCFM reference sequence by more than two nucleotides, and polymorphisms in isolates from the first exposure to MMS (1-1 and 1-2) were rarely preserved in isolates from the third exposure to MMS (3-1 and 3-2).

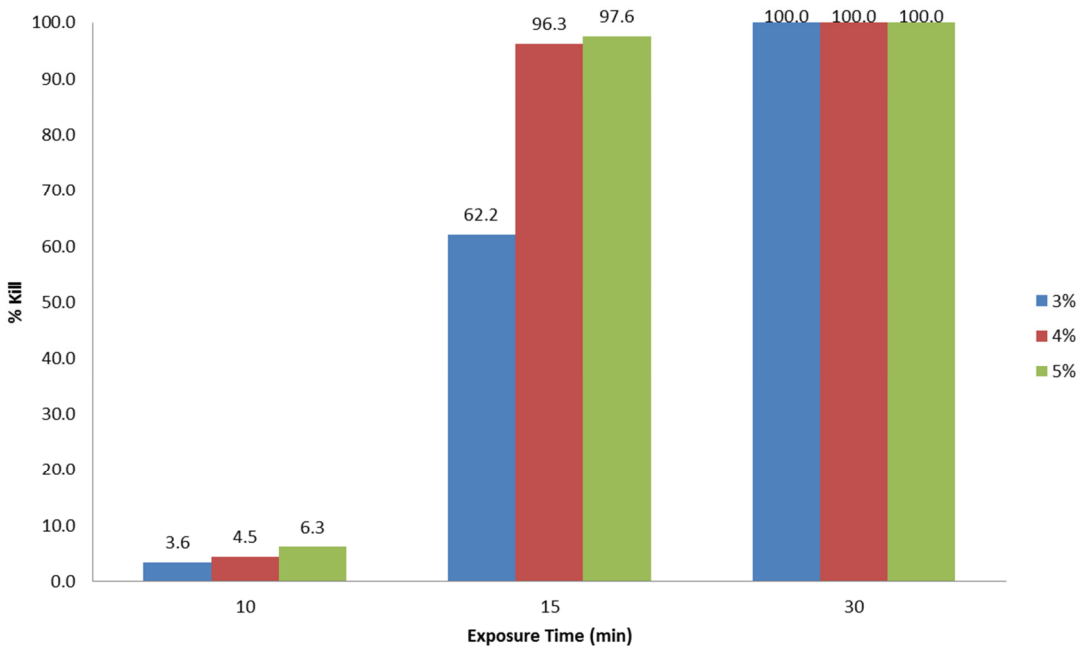


Figure 4.11: The effect of MMS on *L. acidophilus* NCFM at a range of exposure concentrations and times.

The percentage kill due to different MMS concentrations and exposure times is plotted as a bar chart.

Table 4.4: Genome sequences generated for mutagenized *L. acidophilus* NCFM isolates

	Species	Isolate	Year	Isolate source	Sequence source	Genome status
Parent isolate	*L. acidophilus*	NCFM	1970	Derived from LMG 9433	This study	draft
Mutagenised isolates	*L. acidophilus*	NCFM 1-1	2013	Mutagenesis of *L. acidophilus* NCFM – 1st round	This study	draft
	L. acidophilus	NCFM 1-2	2013	Mutagenesis of *L. acidophilus* NCFM – 1st round	This study	draft
	L. acidophilus	NCFM 2-1	2013	Mutagenesis of *L. acidophilus* NCFM – 2nd round	This study	draft
	L. acidophilus	NCFM 2-4	2013	Mutagenesis of *L. acidophilus* NCFM – 2nd round	This study	draft
	L. acidophilus	NCFM 3-1	2013	Mutagenesis of *L. acidophilus* NCFM – 3rd round	This study	draft
	L. acidophilus	NCFM 3-2	2013	Mutagenesis of *L. acidophilus* NCFM – 3rd round	This study	draft

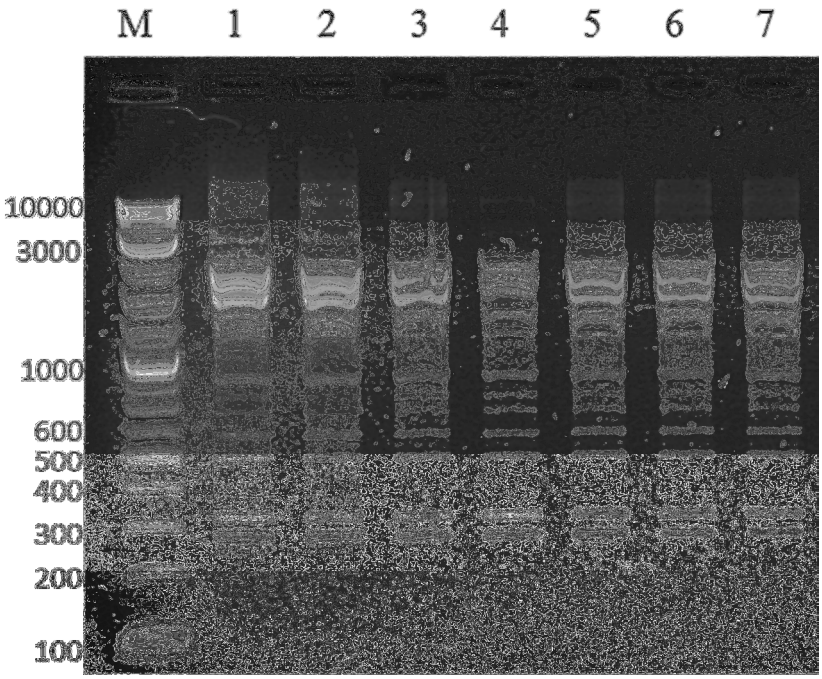


Figure 4.12: RAPD profiles of mutagenized *L. acidophilus* NCFM isolates

RAPD-PCR products from *L. acidophilus* NCFM (lanes 1), and mutagenised *L. acidophilus* NCFM 1-1 (lane 2), 1-2 (lane 3), 2-1 (lane 4), 2-4 (lane 5), 3-1 (lane 6) and 3-2 (lane 7) a negative PCR water control (lane 12) and *L. acidophilus* NCFM DNA as a positive control (lane 13). Lane M contains molecular size marker with sizes of relevant bands given in bp.

Table 4.5: Sequence changes in polymorphic loci from *L. acidophilus* NCFM isolates exposed to 5% (v/v) MMS for 10 minutes

		Sequence change in mutagenised isolates (bp)					
	Mutagenesis:	**1st Round**		**2nd Round**		**3rd Round**	
Locus	**Locus size**	**NCFM 1-1**	**NCFM 1-2**	**NCFM 2-1**	**NCFM 2-4**	**NCFM 3-1**	**NCFM 3-2**
LBA0058	2835	-	-	-	-	-	1
LBA0121	747	-	-	-	-	-	1
LBA0130	453	1	1	1	1	1	-
LBA0236	843	-	-	-	-	-	1
LBA0238	630	-	-	-	-	-	1
LBA0250	705	-	-	-	-	-	1
LBA0326	189	-	-	-	-	1	-
LBA0414	1239	-	-	-	-	1	-
LBA0636	1572	-	-	-	-	-	1
LBA0697	1032	-	-	-	-	-	1
LBA0725	1992	-	-	-	-	-	1
LBA1019	7953	-	-	-	-	-	1
LBA1048	603	-	-	-	-	-	1
LBA1106	564	-	-	-	-	-	1
LBA1191	798	-	-	-	-	-	1
LBA1234	2274	-	-	-	-	-	2
LBA1261	4314	-	-	-	-	-	2
LBA1285	429	-	-	-	-	1	-
LBA1350	900	-	-	-	-	-	1
LBA1166	3483	-	-	-	-	-	1
LBA1442	1257	-	-	-	-	-	1
LBA1460	1020	-	-	-	-	-	1
LBA1578	2085	-	-	-	-	-	1
LBA1642	1296	-	-	-	-	-	1
LBA1788	210	-	-	-	-	-	1
LBA1789	540	-	-	-	-	1	-
LBA1799	1323	-	-	-	-	-	1
LBA1817	750	-	-	-	-	-	1
LBA1939	2340	-	-	-	-	-	1
LBA1165	3624	-	-	-	-	-	1
LBA1166	3483	1	1	1	1	1	-
LBA0238	630	-	-	1	-	1	-
LBA1926	1515	-	-	-	-	-	1
LBA0278	2157	-	-	-	-	-	1
LBA0582	657	1	1	1	1	1	-
LBA0516	702	-	-	-	-	-	1
LBA1382	957	-	-	-	-	-	1
LBA0626	1158	1	1	1	1	1	-
	Total	**4**	**4**	**5**	**4**	**9**	**31**

4.4. DISCUSSION

Genome resequencing analysis was successful in uncovering variation within *L. acidophilus* isolates which was not observed by RAPD as a low resolution pattern matching typing technique. It also illustrated how both the ability to generate genome sequences and analyse them has progressed in a relatively short space of time within this PhD study. Although genome resequencing corroborated the findings of RAPD that indicated *L. acidophilus* was a highly clonal species, it was able to reveal that commercial isolates in current use show even less diversity than other more "historical" isolates from recognised culture collections. Variation in the PAU regions selected as species-specific markers was also observed as well as intriguing differences in the CRISPR regions. The significance of these findings is discussed below.

4.4.1. SMALL SCALE *L. ACIDOPHILUS* GENOMIC DIVERSITY SURVEY

The genomes of two commercial isolates, *L. acidophilus* CUL21 and CUL60 were resequenced and reads were mapped to the *L. acidophilus* NCFM reference sequence. The whole genome was the unit of comparison in this instance, and polymorphism was measured at the single nucleotide level. The level of genomic identity conserved between these commercial isolates and *L. acidophilus* NCFM was exceptionally high. A recent study (Stahl and Barrangou, 2013) yielded similar levels of identity when recently applied to *L. acidophilus* La-14, a commercial isolate used in probiotic products produced by Danisco (Copenhagen, Denmark). Overall, 95 SNPs were discovered between *L. acidophilus* La-14 and *L. acidophilus* NCFM, similar to the 87 and 85 SNPs observed in this study between *L. acidophilus* NCFM and *L. acidophilus* CUL21 and CUL60 respectively (Stahl and Barrangou, 2013). The inclusion of the genome sequence of *L. acidophilus* LMG 11428, a culture collection isolate, showed more overall polymorphisms, but whole genome phylogenies were only able to resolve *L. acidophilus* isolates when only *L. acidophilus* genome sequences were compared (Figure 4.3).

When examining diversity at the scale of the whole genome sequence, highly related sequences such as those generated by *L. acidophilus* isolates present a particular problem. The polymorphic regions, which are the important areas from the point of view of a genomic diversity study, are lost against the backdrop of overwhelming sequence identity. Purely phylogenetic information can be extracted from these SNPs, and phylogenetic analyses built on sequences composed of solely polymorphic positions, as in pathogenic *Salmonella*, where SNP information was used to inform epidemiology (Okoro et al., 2012). The subsequent large scale *L. acidophilus* study, as an alternative to SNP mapping, took a more functional approach to examining genomic diversity by examining each protein coding locus defined in a reference genome sequence (*L. acidophilus* NCFM).

4.4.2. LARGE SCALE L. *ACIDOPHILUS* GENOMIC DIVERSITY SURVEY

4.4.2.1. Gene-by-gene analysis approach

4.4.2.1.1. RMLST OF *L. ACIDOPHILUS* GROUP ISOLATES

Indexing the variation of 53 *rps* loci, which are shared and functionally conserved amongst all members of the domain (Roberts et al., 2008) allows a natural combination and extension of both 16S rRNA gene sequence comparison and traditional MLST to provide resolution from the strain to the domain level (Jolley et al., 2012). Despite the overall genomic diversity within the *L. acidophilus* group, the 16S rRNA gene sequences between some of its members are highly conserved (Bull et al., 2012). This makes the *L. acidophilus* group an ideal candidate for testing the resolution of the rMLST approach, as the 16S rRNA gene phylogenetic trees traditionally used to study evolutionary history are unstable because of their level of sequence conservation (Figure 1.2). A simple Neighbour Joining (NJ) phylogenetic tree based on the sequences of the *rps* genes was able to group isolates of all species together and illustrated the strain-level variation in *rps* genes seen in other *Lactobacillus* species (Figure 4.4). The phylogeny was also better supported than the 16S rRNA gene-based NJ phylogeny drawn from similar isolates (Figure 1.2), suggesting rMLST could provide a greater insight into the evolutionary history of the genus *Lactobacillus* than simple 16S rRNA phylogenies.

4.4.2.1.2. GENOME-WIDE ANALYSES

Numerous studies have used comparative genomics to identify genomic similarities and differences within the LAB (Makarova et al., 2006, Coenye and Vandamme, 2003, O'Sullivan et al., 2009) and at the species level within the *L. acidophilus* group (Berger et al., 2007). No single study however, has conducted a comparative genomics analysis encompassing such a large number of LAB isolates below the species level. This should be considered a fundamental gap in knowledge concerning probiotic bacteria, as probiotic characteristics in many species are unique to a single strain (see Section 2.1). Elucidating the underlying genomic foundations for probiotic phenotypes should be a key target for investigating the mode-of-action of bacterial probiotics. As far as we are aware, this study is the first within the genus *Lactobacillus* to use next-generation DNA sequencing technology combined with a functional, gene-by-gene diversity analysis approach to assess the relatedness and intraspecies diversity of a single probiotic species.

4.4.2.2. Variably present loci

Maintenance of prophage regions within a genome has been shown to affect growth rate and other physiological characteristics such as virulence in pathogenic bacteria (Clark et al., 2012). A small number of protein coding loci were variably present in all isolates, with many of these loci found within regions that show homology to phage-genes (Altermann et al., 2005). Examination of protein sequences show PAU1 and PAU2 are the most closely related, with PAU3 significantly different (Altermann et al., 2005). Two conflicting hypotheses for the origin of the PAU regions are proposed, the first that PAU3 evolved in a different organism and was the latest acquisition and the second that PAU3 was the most ancient integration event and subsequently underwent a duplication event to form PAU1 (Altermann et al., 2005). The presence of PAU1 in *L. acidophilus* LMG 11466, LMG 11467 and LMG 11469, which show no evidence of the presence of PAU3, suggests that PAU3 did indeed evolve in a different organism and was the most recent integration event. There is evidence that PAU region functional homologs exist in other LAB (Altermann and Klaenhammer, 2011). *L. acidophilus* LMG 11466 and LMG 11469 were both shown to lack the region targeted by the *L. acidophilus* specific marker PCR developed in Chapter 2, confirming the negative result shown in Table 2.4.

Generally, loci annotated in the *L. acidophilus* NCFM genome sequence were found in all commercial isolates, with the exception of *L. acidophilus* CUL21 and C47, which lacked PAU1 associated loci. The same situation was not reflected in the culture collection isolates, which were much more diverse in the loci that were absent in their genomes (Figure 4.8 and Figure 4.9). The commercial isolates were more similar to themselves and *L. acidophilus* NCFM than they were to the culture collection isolates which were largely more diverse. The predicted exoproteomes, i.e. the entire set of bacterial proteins predicted to be found in the extracellular milieu (Desvaux et al., 2009) in all isolates of *L. acidophilus* are highly comparable. That is to say, without considering the impact of individual gene sequence on the physiology and characteristics of a strain, the proteins and hence functions that encoded by each gene in the genome of each strain are largely identical.

4.4.2.3. Diverse protein-coding genomic loci

The commercial and culture collection isolates of *L. acidophilus* share almost all protein coding loci defined in the *L. acidophilus* NCFM genome sequence. Two populations emerge when the sequences of these loci are examined and grouped based on allelic profile similarity. These two groups directly reflect the history of the isolates contained within them; culture collection or commercial. The variable loci of commercial isolates of *L. acidophilus* were more similar in sequence to *L. acidophilus* NCFM and formed a homogenous cluster. Similarly to the distribution of absent loci, culture collection isolates formed a more heterogeneous group, distinct from the commercial isolates. No functional category of loci appeared to be more diverse in the genome sequences of culture collection isolates.

One possible explanation for this is the global propagation, storage and repeated re-use of commercial probiotic isolates of *L. acidophilus*, directly related to *L. acidophilus* NCFM. In a similar case in a different probiotic species, two commercial isolates of *L. casei*, isolated directly from probiotic products produced by different companies were found to share a virtually identical genome sequence, encoding a comparable exoproteome and

displaying just 29 SNPs (Douillard et al., 2013). These data and the results of our study indicated that human practice in terms of the use of probiotic LAB or diary starter cultures may be restricting the "natural" evolution of these bacteria, leading the widespread distribution of highly clonal strains.

4.4.2.4. CRISPR regions

The CRISPR regions of *L. acidophilus* isolates were examined as a non-protein-coding region that is typically diverse at the sub-species level in LAB, for example, when diversity in CRISPR loci was indexed in 124 strains of *Streptococcus thermophilus*, 109 different CRISPR arrangements were observed (Horvath et al., 2008). CRISPR regions are usually referred to as CRISPR-Cas (where Cas stands for CRISPR-associated proteins). The Cas genes are normally located within 1kb of the CRISPR locus. They are predicted to be involved in integration of new spacer DNA (Koonin and Makarova, 2009). No Cas genes are present in the *L. acidophilus* genome sequence, which might explain why diversity in the CRISPR region of *L. acidophilus* appears to be driven more by loss of spacer regions rather than acquisition of new spacer regions (Figure 4.10).

L. acidophilus NCFM has a single spacer region that shows 100% sequence identity to its own 16S rRNA gene (Spacer 28, Table 4.3) (Stern et al., 2010). This type self-targeting CRISPR spacer is uncommon, with 0.4% of CRISPR spacers surveyed by Stern *et al* (2010) having incorporated self-genes. The acquisition of self-targeting spacers is also hypothesised to be an accident caused by CRISPR insertion mechanism (Cas genes) and may potentially lead to deleterious effects on the cell (Stern et al., 2010). The fitness cost of self-targeting spacer regions has been demonstrated in *E. coli*, where isolates containing them were seen to have highly degenerated Cas systems (Díez-Villaseñor et al., 2010). Here, a parallel is drawn in *L. acidophilus* where, to alleviate the deleterious effect of a self-targeting CRISPR spacer, *L. acidophilus* has lost all associated Cas genes, and therefore the diversity of these CRISPR regions is not driven by spacer acquisition, specifically those of a self-targeting nature, in this group of isolates.

4.4.3. EXPOSURE OF *L. ACIDOPHILUS* TO MMS

Exposure to 5%(v/v) MMS for 15 minutes was required to kill 99% of *L. acidophilus* NCFM, directly comparable to the Ethyl Methanesulphonate (EMS) exposure conditions required to kill 99% of *L. delbrueckii* subsp. *delbrueckii* ATCC 9649 (4.5%(v/v) for 15 minutes) (Demirci and Pometto, 1992). In *L. delbrueckii* subsp. *delbrueckii* ATCC 9649, no attempt was made to establish the genetic impact of the mutagenesis procedure. Demirci (1992) selected for a mutants that were more tolerant to (d-)-lactic acid by coupling the exposure to EMS with exposure to levels of (d-)-lactic acid above the minimum inhibitory concentration seen in the wild type isolate. This stabilised the random mutagenesis procedure and resulted in an induced, measurable phenotype in their mutants (Demirci and Pometto, 1992).

L. acidophilus NCFM mutants generated in this study were random with no stabilising pressure to enhance a particular phenotype. No observable differences were detected between the RAPD profiles generated by the MMS exposed isolates and the parent *L. acidophilus* NCFM isolate, although this was not unexpected as RAPD is a particularly granular method for examining genomic diversity. This was reinforced when the genome sequences of the MMS exposed isolates showed just 38 polymorphic loci across all exposed isolates, with polymorphic sequence less than two nucleotides in every case. It should be noted however, that this analysis only analysed coding regions of the *L. acidophilus* genome, and mutagenesis is under greater selective pressure in these regions than the intergenic sequence (Lobry and Sueoka, 2002). However, this variation after a few culture passages in the presence of mutagen does demonstrate that *L. acidophilus* may evolve rapidly as a result of mutation. It re-enforces the hypothesis that the stability seen in commercial isolates is a result of derivation from a single clone which has been kept stable as a result of industry practice.

4.4.4. SUMMARY AND PERSPECTIVE

The coding capacity displayed by the *L. acidophilus* isolates examined in this study is highly clonal, with 98% of the protein coding loci found in *L. acidophilus* NCFM present in all other isolates tested. This group of organisms provides a unique opportunity to examine whether diverse probiotic characteristics are a function of genes that are present or absent or, if a level playing field of genes are present, very small changes in the sequence of those genes have a marked effect on the overall probiotic capacity of an isolate. Our study presents an interesting challenge for the bacterial species concept (Fraser et al., 2009), and whether or not bacterial "strains" meaningfully exist as discreet units of sequence diversity in the wider continuum of bacterial species. Although as taxonomic units they may display an array of different sequence types, bacterial strains and species should be defined by measurable and stable phenotypic characteristics. If minor variations in sequence truly influence probiotic effect, low resolution measures of strain type, such as RAPD profile or PFGE profile, are not sufficient to identify a strain. This means that future identification of a probiotic strain of *L. acidophilus* should be based at least on the sequence of a polymorphic genomic locus, or more likely on a whole genome sequence.

In addition, commercially marketing a particular strain of *L. acidophilus* should be dependent on a unique phenotypic characteristic. At this stage however, the homogeneity of the genome sequences of commercial isolates of *L. acidophilus,* also provides companies with the opportunity to assume that evidence of probiotic effect demonstrated in studies undertaken on one particular isolate of *L. acidophilus* would support their own strains equally as well. The demonstration of unique or enhanced probiotic ability in a single isolate will set it apart from the others, and this cannot be achieved in any meaningful way until detailed phenotypic studies encompassing the diversity of isolates seen in this study are conducted.

4.5. CONCLUSIONS

The conclusions from this chapter are as follows:

1. rMLST (*rps*) gene-based phylogenies are a more effective and stable method for establishing phylogenetic relationships within the *L. acidophilus* group than traditional 16S rRNA gene phylogenies.

2. Variably present genomic loci were dominated by genes associated with phage-related regions (PAU). PAU1 was the most widely distributed phage related region, PAU2 and PAU3 were present in all commercial isolates but only some culture collection isolates.

3. Overall, the sequences of 70% of the protein coding loci defined in *L. acidophilus* NCFM were highly conserved in all other *L. acidophilus*, from both commercial applications and culture collections.

4. When genomic diversity was indexed at all protein-coding loci defined in the *L. acidophilus* NCFM genome, two clear divisions of isolates were observed, clearly reflecting whether the isolate had come from a commercial or culture collection background. Commercial *L. acidophilus* isolates formed a more homogenous group than the more diverse culture collection isolates. The homogeneity of the group of *L. acidophilus* isolates of commercial origin suggests that they are a single clone propagated for used in probiotic products globally.

5. When exposed to levels of a chemical mutagen seen to cause mutations detectable as phenotypes in *L. delbrueckii*, analysis of the genome sequences of MMS exposed *L. acidophilus* NCFM showed 38 polymorphic loci that were shared between six isolates. This demonstrated that *L. acidophilus* was capable of genomic change as a result of mutation, and suggests that the homogeneity seen in commercial isolates is a result of their industrial heritage and propagation practice.

5. PHENOTYPIC DIVERSITY OF *LACTOBACILLUS* ISOLATES

5.1. INTRODUCTION

A range of biochemical, physiological, chemotaxonomic, and more recently, nucleic-acid-based methods such as 16S rRNA gene sequence analysis and multilocus sequence typing (MLST) comprise the set of tools useful for obtaining a polyphasic identification and characterisation of both novel and known bacterial isolates (Sintchenko et al., 2007). Historically, the phenotypic methods for characterisation have informed today's bacterial taxonomy. The advent of modern rapid, cost effective and highly accurate methods for analysing nucleic acids has caused phenotypic methods for characterising bacteria to take somewhat of a back seat in typing, identification and taxonomy. The application of techniques for accurately and rapidly identifying and characterising bacteria however, is essential to numerous fields in microbiology (De Bruyne et al., 2011). From a commercial probiotic perspective it is important to select bacterial isolates that have desirable phenotypic characteristics, particularly the ability to grow to high densities in as little time as possible.

The characterisation of bacterial isolates by profiling their ability to ferment carbohydrate substrates has long been a staple method of distinguishing between LAB, and indeed forms the basis of the primary divisor of LAB into those that are obligately homofermentative, those that are obligately heterofermentative and those that are facultatively heterofermentative (Hammes and Vogel, 1995). The API 50 CH test evaluates the ability of isolates to ferment 50 carbohydrate substrates, allowing discrimination up to the species level when compared with a database of known biochemical profiles (Boyd et al., 2005).

Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) is a novel rapid and high-throughput method, that has been recently introduced as a tool for bacterial taxonomy and characterisation, and has been applied to a number of taxa (Zhu et al., 2013) (Kern et al., 2013), including lactobacilli (Angelakis et al., 2011)

(Dušková et al., 2012). MALDI-TOF MS has the ability to analyse complex peptide mixtures, such as intact bacterial cells or total protein extracts from bacterial cultures.

Previous chapters have indexed the diversity of *Lactobacillus,* particularly *L. acidophilus* at the nucleic acid level, finding little variation between *L. acidophilus* isolates. In this chapter the phenotypic diversity of *L. acidophilus* was examined at multiple taxonomic levels. At the species level, carbohydrate fermentation characteristics were evaluated, and at the isolate level growth parameters and gross proteomes were compared between isolates.

5.1.1. SPECIFIC AIMS

The aims of this chapter were as follows:

- Examine species-level differences in *Lactobacillus* in the metabolism of carbohydrates using API 50CHL and evaluate it as a method for identifying *L. acidophilus*.

- Measure parameters of growth (length of lag phase, maximum growth rate and maximum culture density) of *L. acidophilus* to determine if isolate to isolate differences exist.

- Evaluate the use of Matrix Assisted Laser Desorption/Ionisation – Time Of Flight (MALDI-TOF) mass spectrometry as a tool to examine the gross proteome of *Lactobacillus* isolates.

- Assess the use of MALDI-TOF MS as an identification tool for *Lactobacillus* isolates on both the species and strain levels.

5.2. METHODS

5.2.1. EVALUATION OF *LACTOBACILLUS* CARBOHYDRATE FERMENTATION PROFILES USING THE API 50CHL SYSTEM

Lactobacillus isolates were grown on MRS agar at 37°C for 24 h, as described previously. As per the manufacturer's instructions, single colonies from each culture were suspended in API 50 CHL medium (BioMérieux, France). The suspension was transferred into API 50 CH strips (BioMérieux, France). All wells were overlaid with sterile mineral oil ensure anaerobic metabolism. API strips were incubated at 37°C as recommended by the manufacturer. Changes in the colour of wells were recorded after 24 and 48 h. API test kit results were interpreted using the Analytical Profile Index (API) database of the apiweb™ software (version 4.0; BioMérieux, Marcy l'Etoile, France).

5.2.2. MEASURING *LACTOBACILLUS* GROWTH RATES

5.2.2.1. Bioscreen C

The growth dynamics of *L. acidophilus* isolates LMG 11470, LMG 11428, LMG 9433, NCFM, CUL 21, CUL60, Rm 344; *L. casei* LMG 6904 and six MMS exposed *L. acidophilus* (see Section 4.2.5) were examined using a Bioscreen Microbiolgical Growth analyser C (Labsystems, Finland). *Lactobacillus* isolates were cultured as previously described (Section 2.2.2). 3 ml overnight (18 h) cultures were diluted to an optical density of 1±0.2 (600nm), then diluted 10-fold and transferred to triplicate wells of a Bioscreen microplate (200 µl). Growth analysis was performed for 48 hours at 37°C; turbidity measurements were taken at 15 minute intervals using a wide-band filter (450-580 nm), after shaking the microplates for 10 seconds at an intermediate intensity. Experiments were repeated with different starting cultures to obtain two biological replicates with a combined total of six technical replicates.

5.2.2.2. Estimation of growth parameters

Before analysis, to prepare the data, the mean optical density of triplicate blank wells (with no inoculum) was subtracted from each test well and growth curves were trimmed to 20 h to ensure that curves were fitted correctly. Growth parameters were then estimated using the gcFit function within the grofit package (Kahm et al., 2010) in R statistical software (R Development Core Team, 2012). Briefly, a model-free spline was fitted to logarithmically transformed optical density data (solid red line, Figure 5.1) and visually checked for accuracy. Any wells that generated poorly fitted splines were discarded from further analysis. Accurately placed splines were then used to estimate three growth parameters for each well; length of lag phase, maximum growth rate and maximum culture density reached (Figure 5.1). All of the above parameters were calculated for each well and exported from the software.

5.2.2.3. Comparing growth parameters

Growth parameters generated by *Lactobacillus* isolates were grouped, as in Chapter 4, into six groups; *L. acidophilus* culture collection isolates (LMG 11470, LMG 11428 and LMG 9433), *L. acidophilus* commercial isolates (NCFM, CUL 21, CUL60 and Rm 344), non- *L. acidophilus* isolates (*L. casei* LMG 6904) and three groups of MMS exposed *L. acidophilus* NCFM isolates, one from each round of exposure to MMS (see Section 4.2.5). Boxplots were generated using the boxplot function in R statistical software (R Development Core Team, 2012).

Data were tested for normality using the Shapiro-Wilk test in R statistical software (R Development Core Team, 2012). Significant differences between groups were investigated using the Kruskall-Wallis H-test followed by pairwise, post-hoc Mann-Whitney U tests with the familywise error rate controlled using Bonferroni correction in IBM Statistics 20 (IBM Corporation, New York, US).

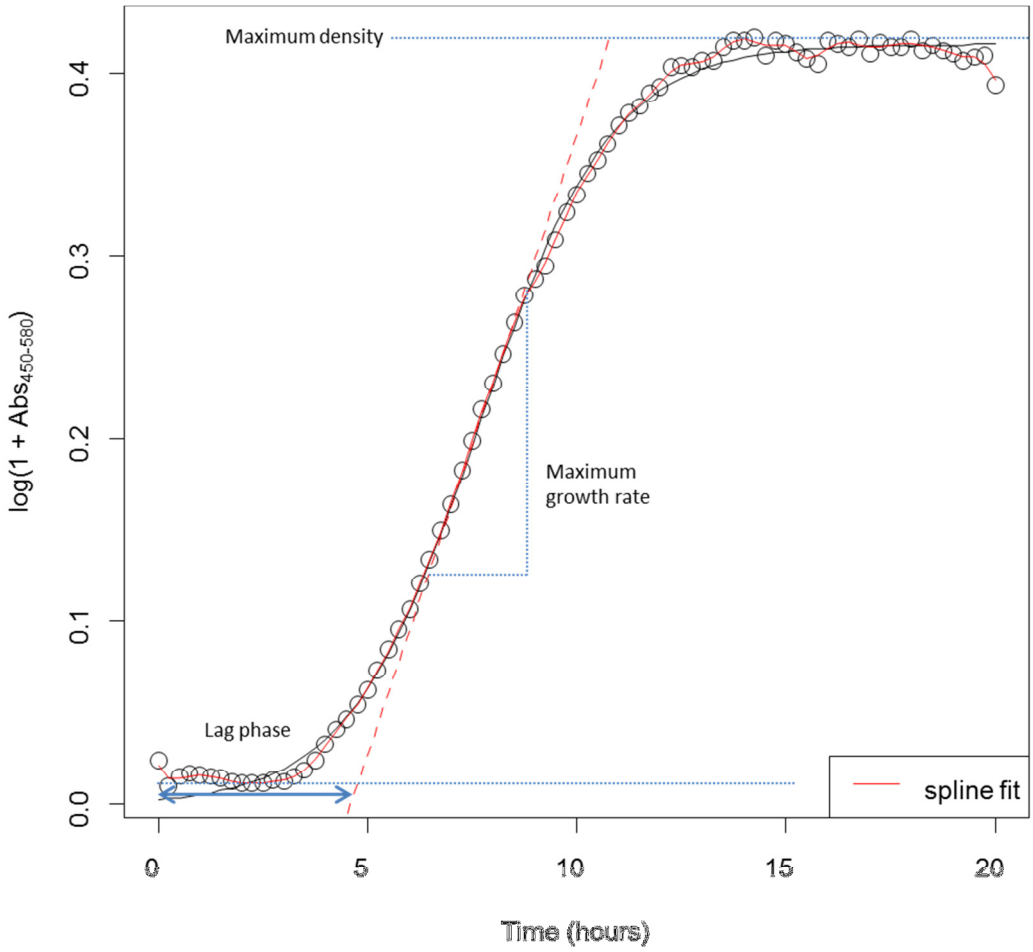


Figure 5.1: Three growth parameters calculated using grofit

The three measured parameters are indicated with blue lines and describe the length of the lag phase, the maximum growth rate and the maximum culture density reached in each culture.

5.2.3. GROSS PROTEOME ANALYSIS USING MATRIX-ASSISTED LASER DESORPTION/IONIZATION-TIME-OF-FLIGHT MASS SPECTROMETRY (MALDI-TOF MS)

5.2.3.1. Bacterial strains

Proteomes of *L. acidophilus* isolates and isolates of other LAB were examined with MALDI-TOF MS (Table 5.1). Strains were resuscitated from charcoal transport swabs (Fisher Scientific, UK) onto MRS agar at 37 °C for 24h. After resuscitation, strains were grown anerobically on MRS agar at 37 °C for 24 h and checked for purity. Prior to MALDI-TOF MS analysis, the strains were subcultured at least twice.

5.2.3.2. Sample preparation – "Cell extract"

The crude protein sample preparation protocol is described in detail by De Bruyne *et al* (2011). Briefly, α-cyano-4-hydroxycinnamic acid (CHCA) (5 mg/ml):acetonitrile (ACN):water:trifluoroacetic acid (TFA) are combined in the ratio 50:48:2 to form a matrix-organic solvent mixture as described by Williams *et al* (2003). Chemicals were of high performance liquid chromatography (HPLC) grade quality. 1 μL of bacterial cells (manipulated by Looplast® inoculation loops) was washed in HPLC grade water and ethanol. 70% formic acid and pure ACN were added in a 1:1 (v/v) ratio to the bacterial pellet, and the suspension mixed by vortex for 30 s. The supernatant, obtained after microcentrifugation, was then transferred into a new tube, forming the "cell extract". Cell extracts (1.5 μL) were then transferred to the spot sites on a 384-well stainless steel target plate and air-dried for about 10 min. The matrix–organic solvent mixture (1 μL) was added to the spots and allowed to dry. Each sample was spotted at least in quadruplicate, to verify reproducibility. The samples were allowed to air-dry at room temperature, inserted into the mass spectrometer and subjected to MALDI-TOF MS analysis.

Table 5.1: *Lactobacillus* and *Enterococcus* isolates used in MALDI-TOF proteome analysis

Species	Isolates
L. acidophilus	LMG 11428
	LMG 11430
	LMG 11470
	LMG 13550
	LMG 9433
	CUL21
	CUL60
	NCFM
	RM 344
	RM 345
L. brevis	LMG 6906
L. casei	LMG 6904
L. gasseri	LMG 9203
L. johnsonii	LMG 9436
L. paracasei subsp. paracasei	LMG 7955
L. plantarum	LMG 6907
Enterococcus faecium	LMG 14205

5.2.3.3. MALDI-TOF MS sample analysis

Prior to analysis, the mass spectrometer was externally calibrated with a peptide mix of adrenocorticotropic hormone (ACTH) (18-39), insulin (bovine), ubiquitine, cytochrome c and myoglobin. The matrix solution and external calibration peptide mix were mixed in a 1:1 (v/v) ratio and spotted (1 µl) on the designated calibration spots on the 384-well target plate. The 4800 Plus MALDI TOF/TOF™ Analyzer (Applied Biosystems, Framingham, MA, USA) was used in the linear mode. The mass spectrometer uses a 200-Hz frequency tripled Nd:YAG laser, operating at a wavelength of 355 nm. Ions generated by the MALDI process were accelerated at 20 kV through a grid at 19.3 kV into a linear, field-free drift region and subsequently into the detector. The detector, an electron multiplier, detected and counted the generated ions. For each spot, 40 sub-spectra for each of 50 randomized positions within the spot (2000 spectra/spot) were collected and presented as one main spectrum. MALDI-TOF mass spectra were generated in the mass range 2–20 kDa. Laser intensity was set between 3600 and 3800 V, obtaining signal intensities between 5 × 102 and 1 × 104. Data were collected in an automated fashion using random sampling over the sample spot.

5.2.3.4. Data pre-processing

Raw data were extracted as .t2d files from the 4800 Plus MALDI TOF/TOF™ Analyzer. The t2d files were imported in the Data Explorer 4.0 software (Applied Biosystems, CA, USA) and transformed to text files. These text files consisted of an array containing the signal intensity for each 0.5 mass-to-charge ratio (m/z) value. A Data Explorer script (De Bruyne et al., 2011) was used to export the peak list of each of the samples from one spot set to separate text files, which were used as input files for the BioNumerics 6.0 software package (Applied-Maths, Sint-Martens-Latem, Belgium). In BioNumerics 6.0 a densitometric curve was reconstructed, plotting signal intensity against (m/z). This workflow was integrated into a script to facilitate the import of the normalized peak lists as densitometric curves. Since all measurements were performed after calibration of the 4800 Plus MALDI TOF/TOF™ Analyzer, data could be considered normalized, and additional normalization of the experiment type was not performed. Data were reduced and de-noised using the methods described by De

Bruyne *et al* (2011). The quality of each spectrum was evaluated using the following criteria; minimum signal intensity of 1000 units, less than 30% leading slope into spectrum and more than five peaks. Any spectra not meeting these criteria were removed from further analysis.

5.2.3.5. Data analysis

Similarities between densiometric curves generated by each spot were calculated using curve-based (Pearson Product Moment Correlation Coefficient, PMCC) measures and clustering was initially performed using the unweighted paired-group method with arithmetic mean (UPGMA) method. Multi-dimensional scaling (MDS) was used for the visualization of the likeliness of data, for example, for exploring similarities or dissimilarities. The MDS approach started with a matrix of data similarities generated in BioNumerics 6.0. This was then imported into IBM Statistics 20 and similarities were plotted in 2-dimensional space using a non-linear least squares fit, to minimise the distances between the data points, known as Multidimensional Scaling. The resulting data positions were visualised in a 2-dimensional scatter plot using Microsoft Excel.

5.3. RESULTS

For clarity, the results were divided into three parts, the first (Section 5.3.1) concerns the elucidation of carbohydrate fermentation profiles of *L. acidophilus* isolates. These included two *L. acidophilus* isolates from the Cultech Lab4® probiotic supplement (CUL21 and CUL60), *L. acidophilus* isolates from culture collections, and *L. casei* (*L. acidophilus*) TBCC, which was re-classified from *L. acidophilus* to *L. casei* in Chapter 2 based on its RAPD profile. The second part (Section 5.3.2) describes the comparison of growth characteristics of *L. acidophilus* isolates from culture collections, commercial applications and those that were exposed to the mutagen MMS (Section 4.2.5). The third (Section 5.3.3) compares gross proteomes of *Lactobacillus* isolates using MALDI-TOF MS.

5.3.1. CARBOHYDRATE FERMENTATION BY *LACTOBACILLUS* ISOLATES

The API 50CHL test was used to evaluate the carbohydrate fermentation characteristics of *L. acidophilus* isolates. Figure 5.2 catalogues the ability of eight *L. acidophilus* isolates and *L. casei* TBCC to ferment 20 carbohydrate substrates. The fermentation characteristics of *L. casei* TBCC were markedly different to the *L. acidophilus* isolates tested. *L. casei* TBCC did not ferment D-maltose or sucrose, both of which were fermented by 100% of *L. acidophilus* isolates. *L. casei* TBCC was also able to ferment D-mannitol, arbutin, D-melezitose, D-tagatose and potassium gluconate, none of which were fermented by any *L. acidophilus* isolates (Figure 5.2). The apiweb™ fermentation profile analysis software classified this profile as *L. paracasei* subsp. *paracasei,* although it was still assumed to be a doubtful designation. Among *L. acidophilus* isolates, API 50 CHL profiles showed a large degree of heterogeneity. This was reflected in the apiweb™ classifications for each of the profiles, which classified five isolates as *L. acidophilus* with a good or acceptable profile, two isolates as *L. acidophilus* with doubtful or not valid profiles and a single isolate as *Lactobacillus* to the genus level (Figure 5.2).

Four carbohydrates; D-glucose, Esculin, D-maltose and D-saccharose were fermented by 100% of *L. acidophilus* isolates, with the fermentation of D-maltose and D-saccharose clearly differentiating between *L. casei* TBCC and *L. acidophilus* isolates. D-galactose, D-fructose, D-mannose, salicin and D-trehalose were fermented by 87.5% of *L. acidophilus* isolates, although the lack of ability to ferment all of these substrates was not restricted to a single *L. acidophilus* isolate. *L. acidophilus* CUL 60 was able to ferment the largest array of carbohydrate substrates (15). *L. acidophilus* NCFM and LMG 11470 fermented 14 carbohydrates, LMG 11472 and LMG 11467 fermented 12, LMG 9433 fermented 11, and LMG 11428 and LMG 11466 fermented 10. There was no clear distinction in the fermentation profiles of isolates from culture collections (*L. acidophilus* LMG 11470, LMG 11472, LMG 11467, LMG 9433, LMG 11428 and LMG 11466) and those from commercial applications (*L. acidophilus* NCFM and CUL60).

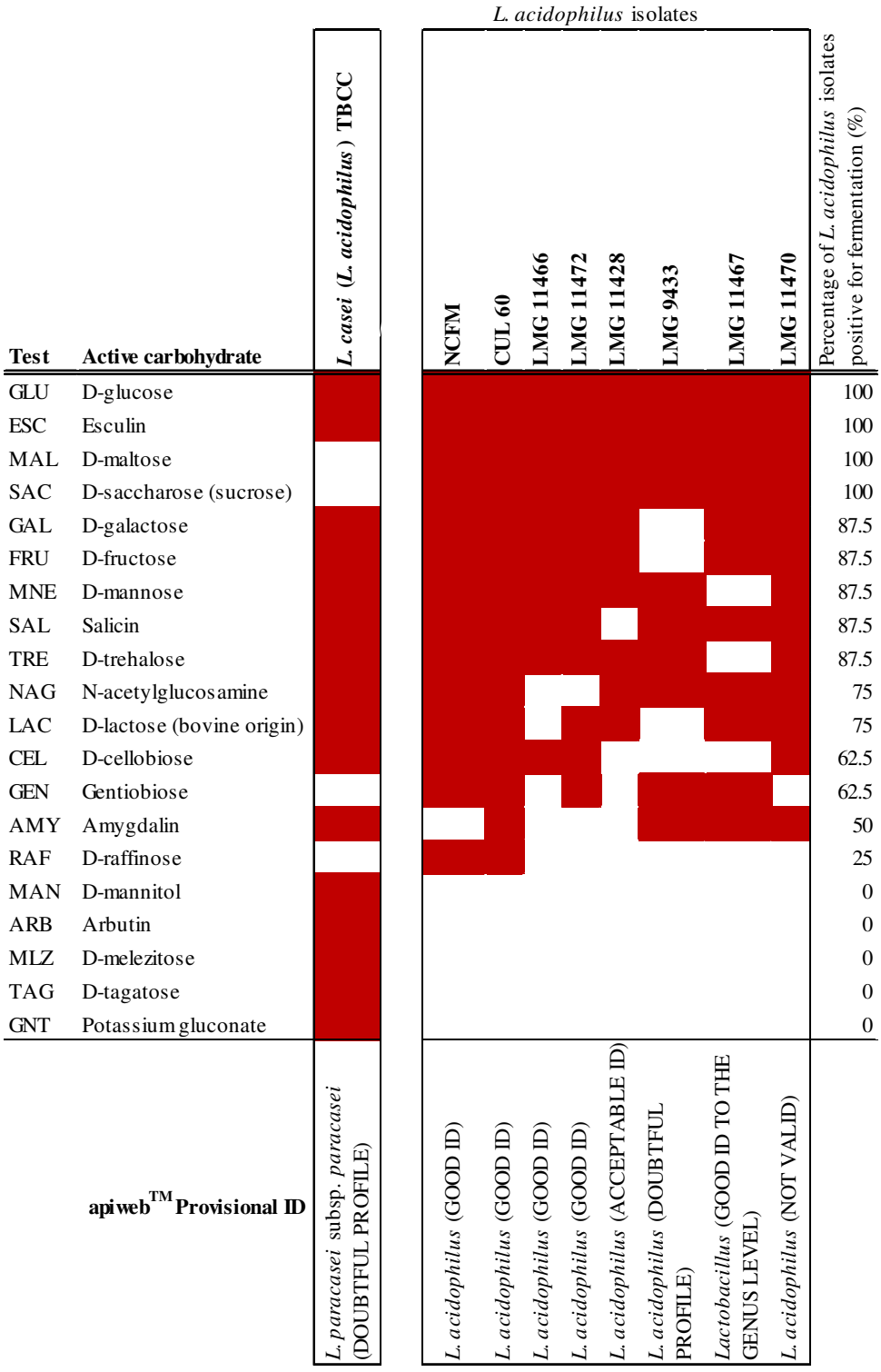


Figure 5.2: Carbohydrate fermentation profiles of *Lactobacillus* isolates

Carbohydrate fermentation profiles from API 50 CHL. Only tests that were positive in one or more isolates are included. Positive tests are coloured red. Indicated provisional identification was generated using apiweb™ software (bioMérieux UK, Basingstoke, UK).

5.3.2. *LACTOBACILLUS* GROWTH CHARACTERISTICS

L. acidophilus isolates were divided into five groups, the first representing culture collection isolates, the second commercial isolates and three groups representing the three rounds of exposure to MMS (see Chapter 4). *L. casei* LMG 6904 was included for comparison, as a non-*L. acidophilus* isolate. The growth kinetics of these groups are represented by three parameters defined in Figure 5.1, which include length of lag phase, maximum growth rate and maximum culture density. The spread of these data is shown in Figure 5.3. None of the data generated for any of the three parameters was normally distributed when tested with the Shapiro-Wilk test, so differences between groups were tested for significance using the non-parametric Kruskall-Wallis H-test, which was corrected for tied ranks, followed by post-hoc pairwise Mann-Whitney U tests with Dunn-Bonferroni correction.

All growth parameters showed significant differences across groups. The Kruskal-Wallis test revealed a significant effect of group on maximum culture density (χ^2=83.51, d.f.=5, $p < 0.01$), maximum growth rate (χ^2=49.39, d.f.=5, $p < 0.01$) and lag time (χ^2=37.37, d.f.=5, $p < 0.01$). Pairwise comparisons of groups for each growth parameter are given in Table 5.2. The non-*L. acidophilus* group grew to significantly higher maximum culture density than all other groups except MMS 3. No significant differences were observed between the culture collection and commercial groups, or between either of the latter groups and the MMS exposed groups. The culture collection group had a significantly lower maximum growth rate than all groups except the commercial group, between which there was no significant difference. Commercial isolates also had a lower maximum growth rate than the non- *L. acidophilus* group and the MMS 1 group. The MMS exposed groups (MMS 1, MMS 2 and MMS 3) had significantly longer lag phases than the culture collection group and the commercial group. No significant difference was observed in the lag phases of the commercial and culture collection groups, or between these groups and the non- *L. acidophilus* group.

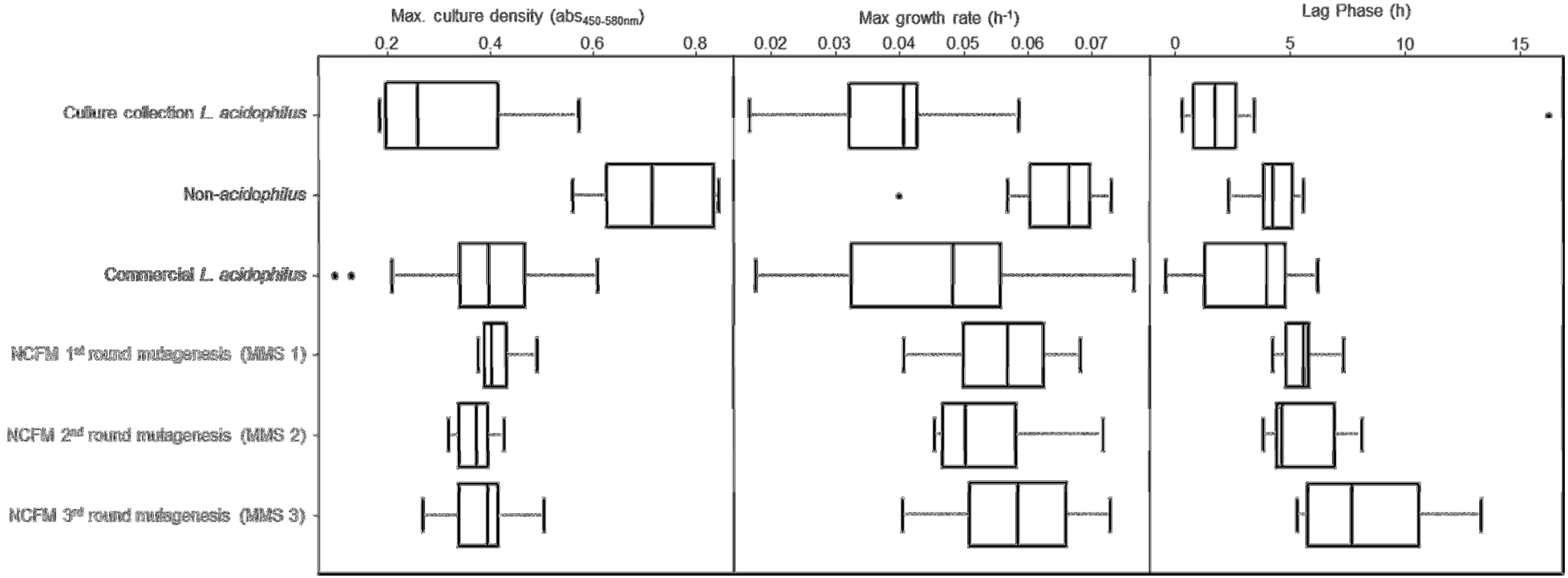


Figure 5.3: Growth parameters of *Lactobacillus* isolates

Growth parameters maximum culture density, maximum growth rate and length of lag phase are defined in Figure 5.1. *Lactobacillus* isolates are divided into groups representing isolates from culture collections (LMG 11470, LMG 11428 and LMG 9433), commercial applications (NCFM, CUL 21, CUL60 and Rm 344), non- *L. acidophilus* (*L. casei* LMG 6904) and isolates from three rounds of mutagenesis with MMS, as described in (Chapter 4). Boxes represent median and inter-quartile range; whiskers extend to 1.5 times the inter-quartile range, or the most extreme datum, whichever is closer to the median. Outliers are shown as circles outside whiskers.

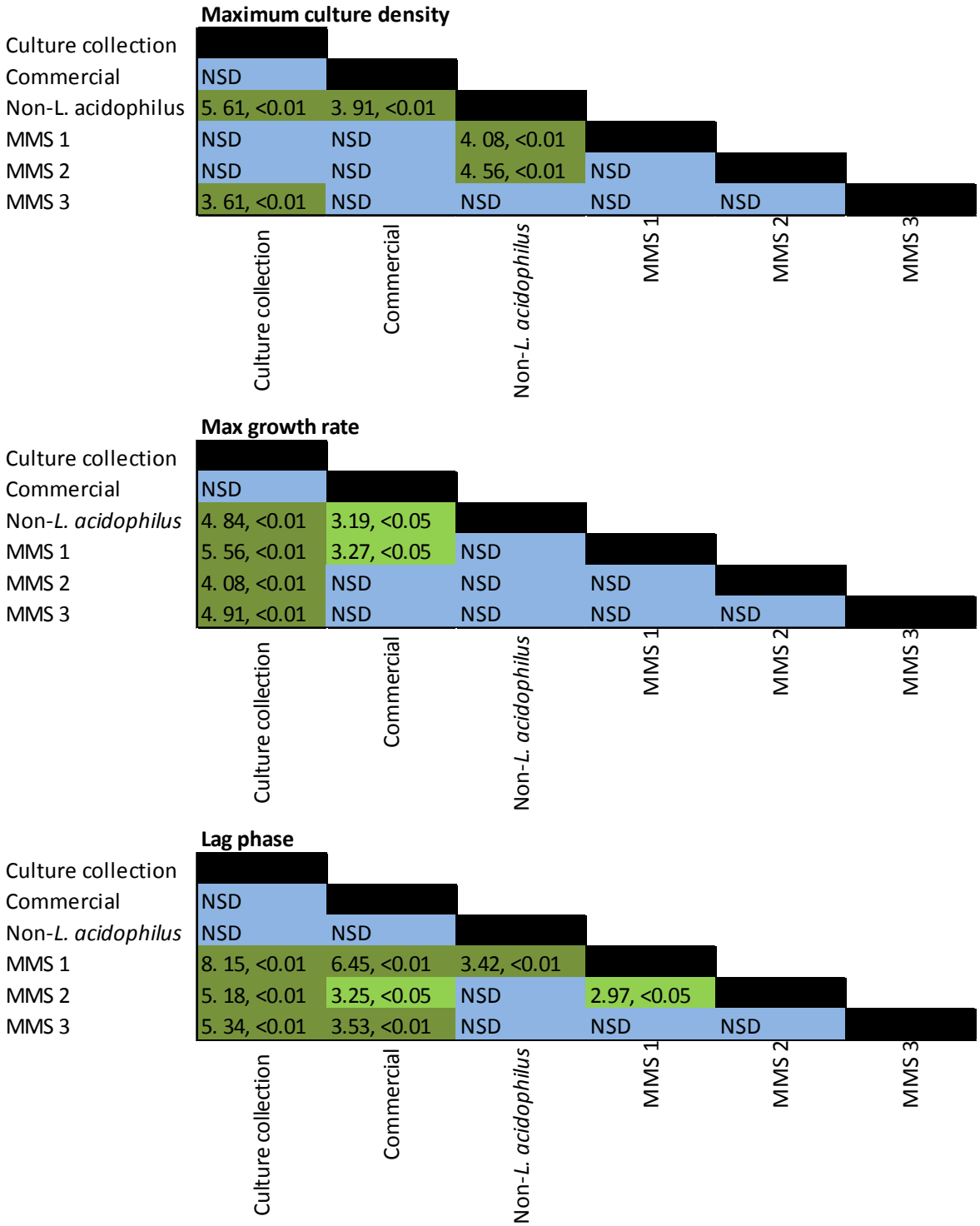


Table 5.2: Significant differences in growth parameters between groups of *Lactobacillus* isolates

Statistics are reported as effect size, followed by significance if greater than 0.05. Comparisons with no significant differences are reported with NSD.

5.3.3. COMPARISON OF THE GROSS PROTEOME OF *LACTOBACILLUS* ISOLATES

MALDI-TOF MS was used to examine the gross proteome of *Lactobacillus* isolates with particular emphasis on *L. acidophilus* isolates. A scaling workflow of data analysis was undertaken, represented by the progression of data quantity displayed in three MALDI-TOF profiles in Figure 5.4, to tens of profiles analysed and displayed in Figure 5.5, to finally hundreds of profile similarities easily visualised in Figure 5.6.

When visualised at the single profile level with no similarity analysis undertaken (Figure 5.4), *L. acidophilus* profiles are visually similar, and the included *L. brevis* profile is markedly different. When examined based on MALDI-TOF profile similarity (Figure 5.5), all *L. acidophilus* isolates cluster together at greater than 81% MALDI-TOF profile similarity. Repeat testing of the same isolates of *L. gasseri* LMG 9203, *L. johnsonii* LMG 9436 and *E. faecium* LMG 14205 show profile stabilities of 95.7%, 94.7% and 94.5% respectively (Figure 5.5). The MALDI-TOF profiles generated by *L. gasseri* LMG 9203 and *L. johnsonii* LMG 9436 show 64.6% similarity to each other (Figure 5.5).

Non-metric multidimensional scaling (nMDS) allows the visualisation of hundreds of MALDI-TOF profiles in low-dimensional (in this case two-dimensional) space. More similar MALDI-TOF profiles are plotted as points with low Euclidian distances between, and more dissimilar MALDI-TOF profiles are plotted more disparately on a set of arbitrary axes. When applied to *Lactobacillus* isolates, this approach visualised the high level of MALDI-TOF profile conservation of repeat tests of the same strain i.e. profiles generated by the same isolate occupy similar space within the nMDS plot (Figure 5.6). *L. gasseri* LMG 9203 and *L. johnsonii* LMG 9436 occupy a similar area on the nMDS plot (Figure 5.6), although their profiles share 64.5 % similarity (Figure 5.5). All other *Lactobacillus* species formed distinct clusters (Figure 5.6). All *L. acidophilus* isolates shared the same area on the nMDS plot, although diversity in profiles can be observed (81.0% profile similarity in Figure 5.5 and distance between points in (Figure 5.6). To reflect the two groups of *L. acidophilus* genotypes observed in Chapter 4 and Section 5.3.2, the MALDI-TOF nMDS plot was coloured to represent culture collection and commercial *L. acidophilus*. No clear distinction was seen in the graph area occupied by these two groups.

This indicates that the diversity of *L. acidophilus* MALDI-TOF profiles generated by *L. acidophilus* isolates cannot be explained by differences between these two groups.

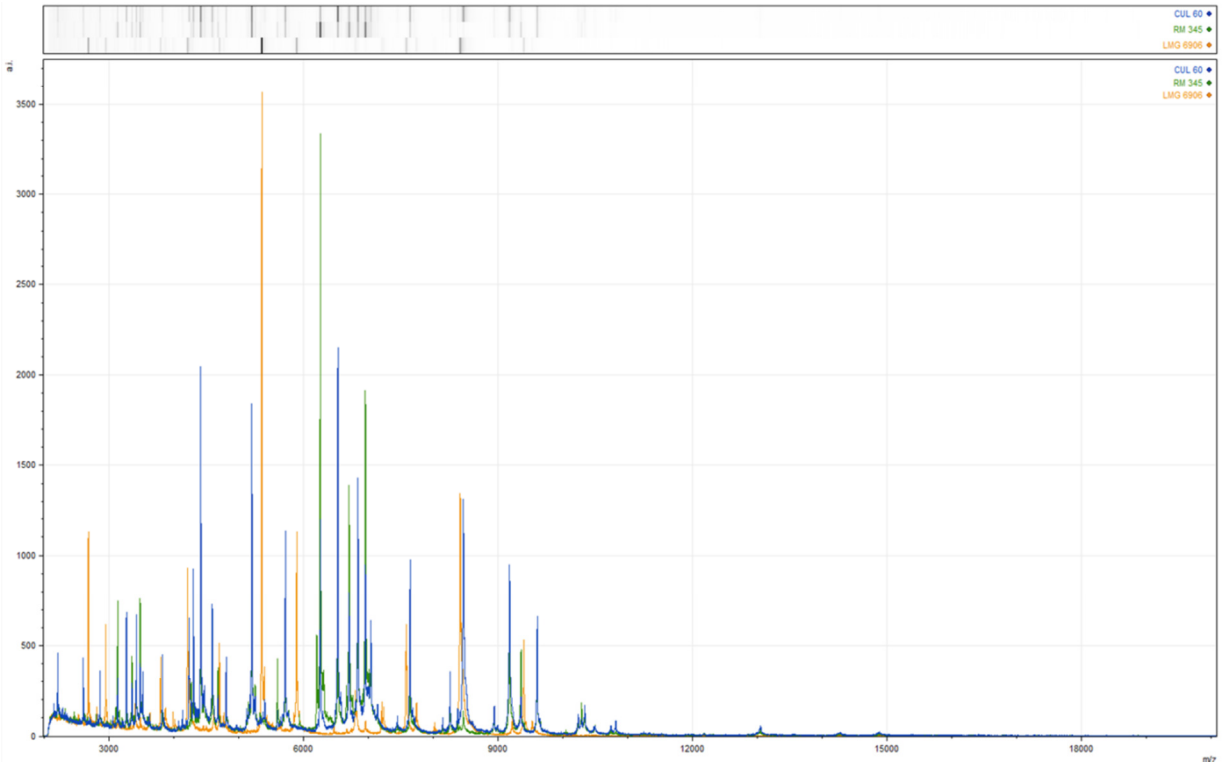
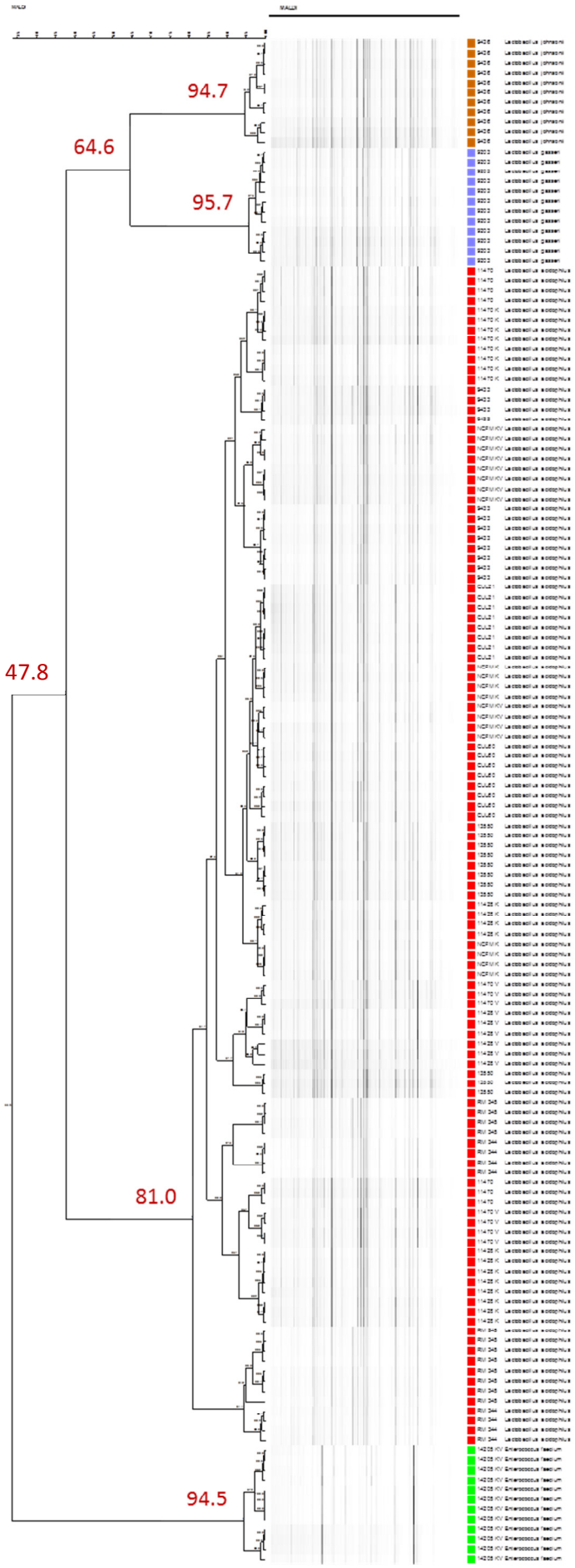


Figure 5.4: MALDI-TOF spectra of three *Lactobacillus* isolates and their relation to MALDI-TOF fingerprints

Signal intensity (y-axis) is plotted against m/z (x-axis). MALDI-TOF profiles are given for three *Lactobacillus* isolates; *L. acidophilus* CUL 60 (blue), *L. acidophilus* Rm 345 (green) and *L. brevis* LMG 6906 (orange). Their corresponding MALDI-TOF fingerprints are given above the spectra, with peaks of greater intensity represented as darker bands.


94.7
64.6
95.7
47.8
81.0
94.5

← ***Previous page***

Figure 5.5: Clustered MALDI-TOF profiles generated by *Lactobacillus* isolates

L. johnsonii (orange), *L. gasseri* (blue), *L. acidophilus* (red) and *E. faecium* (green) MALDI-TOF profiles were clustered using BioNumerics 6.0 (Applied Maths, Belgium). Pearson correlation similarity coefficient with a UPGMA dendrogram type was used, and position tolerance optimisation was set to 2%. Pertinent profile similarity values are indicated in red text adjacent to the corresponding node.

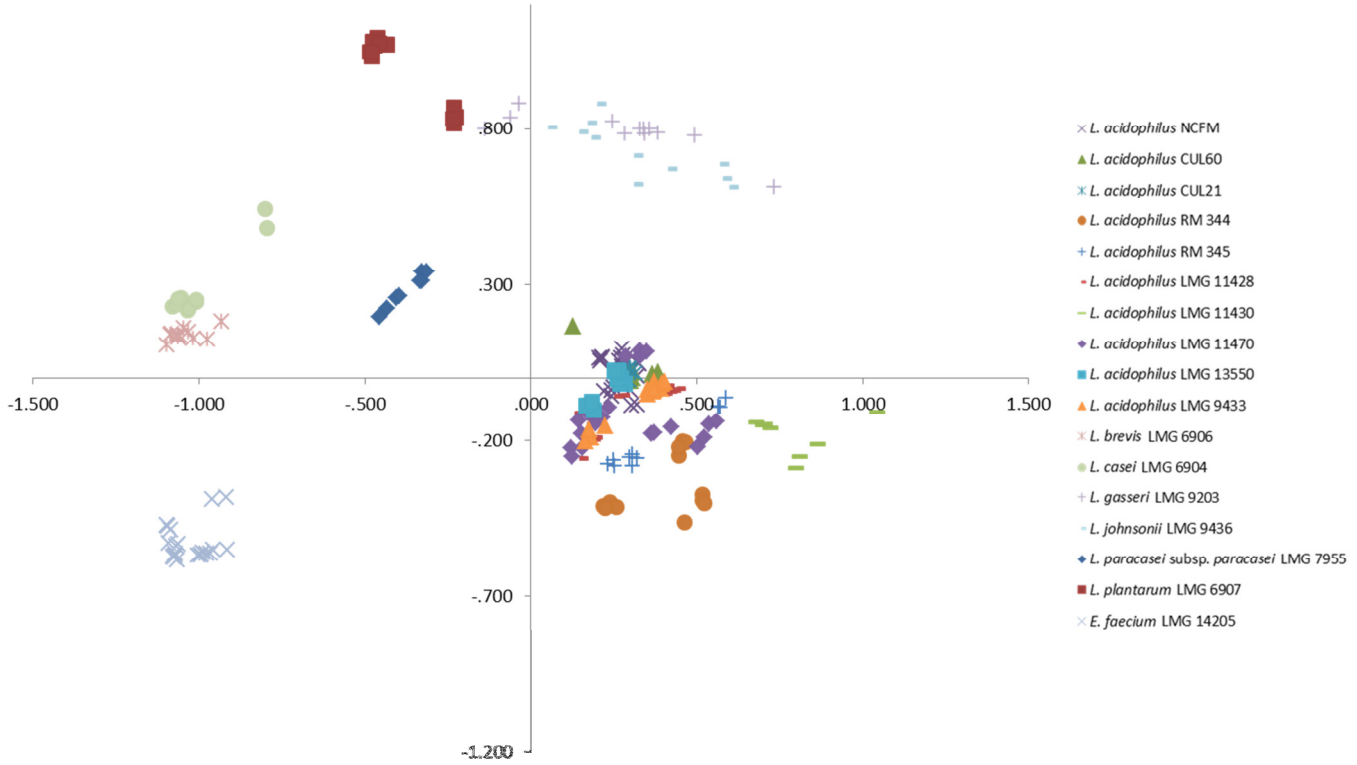


Figure 5.6: Diversity of *Lactobacillus* MALDI-TOF profiles

MALDI-TOF profile distance scores are plotted in two dimensions, axes are arbitrary. Distances between profiles are represented by Euclidian distance, i.e. more similar profiles are plotted as points closer together. Distances were calculated using the Pearson correlation similarity coefficient and position tolerance optimisation was set to 2%. Coordinates were calculated using multidimensional scaling. .

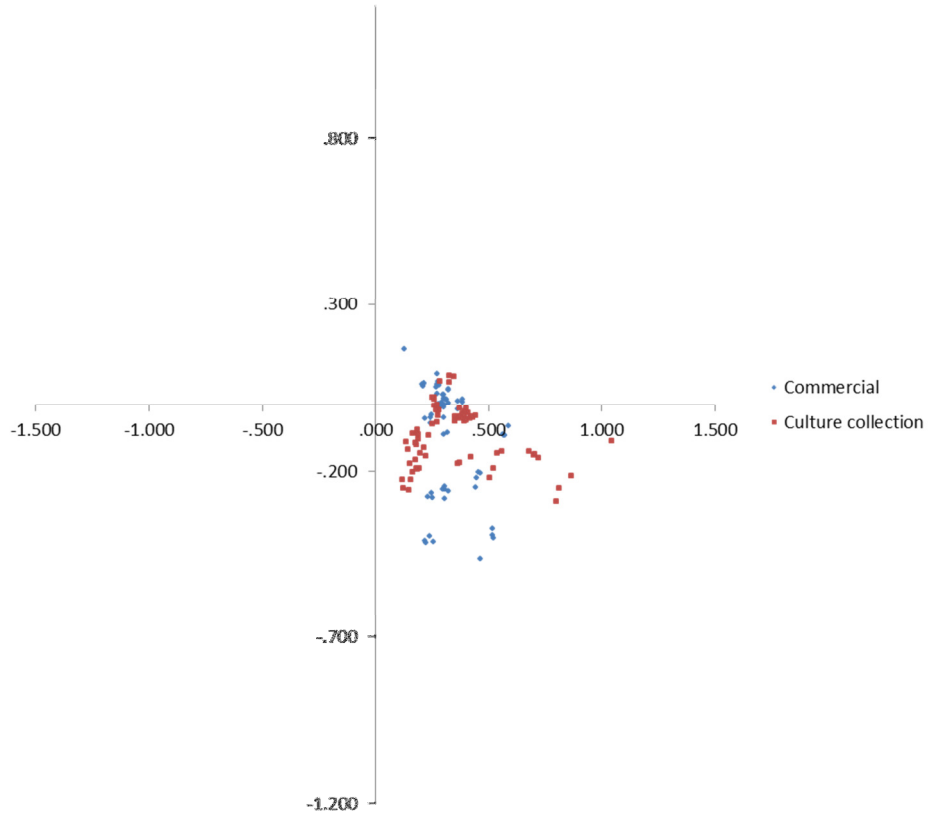


Figure 5.7: Diversity of *L. acidophilus* MALDI-TOF profiles

MALDI-TOF profile distance scores are plotted in two dimensions, axes are arbitrary, as previous (Figure 5.6). *L. acidophilus* isolates were grouped into commercial and culture collection isolates as described previously (see Chapter 4). Distances were calculated using the Pearson correlation similarity coefficient and position tolerance optimisation was set to 2%. Coordinates were calculated using multidimensional scaling.

5.4. DISCUSSION

5.4.1. CARBOHYDRATE FERMENTATION PROFILES OF *L. ACIDOPHILUS* ISOLATES

A diverse range of carbohydrate fermentation characteristics were observed within *L. acidophilus*, and identifications with the associated database (apiweb™) varied in veracity and taxonomic-level discrimination. A previous, comprehensive study of the diversity within the *L. acidophilus* group using a polyphasic approach used API as a part of that approach. Two distinct fermentation profiles for two *L. acidophilus* isolates (NCFM and ATCC 11975) that had been thoroughly characterised using comparative nucleic acid sequences at all levels from single functional genes to whole genomes were found (Berger et al., 2007). The degree of variability in API fermentation profiles displayed by *L. acidophilus* isolates in this study and previously (Berger et al., 2007), means that it is difficult to identify isolates based on carbohydrate fermentation profile alone and indeed the European Union-funded "Biosafety Assessment of Probiotics used for Human Consumption" (PROSAFE) project recommend that biochemical systems (such as API 50 CHL) should not be used as a stand-alone approach for identification of probiotic cultures. These systems may be useful to obtain a first tentative classification at the genus level in conjunction with primary phenotypic tests, but the identification result should in any case be confirmed by other (molecular) methods, forming the basis of the polyphasic taxonomy required for accurate identification and typing of LAB (Vankerckhoven et al., 2008). The value of polyphasic typing methods is exemplified in the case of *L. casei* (*L. acidophilus*) TBCC, whose partial 16S rRNA gene sequence was not sufficient to identify the isolate correctly. Comparative analysis of carbohydrate fermentation ability in this chapter (Figure 5.2) and RAPD profile (see Chapter 2) have shown that this isolate does not belong to *L. acidophilus*. It was doubtfully classified as *L. paracasei* subsp. *paracasei* by apiweb™, did not produce a RAPD profile that was similar to *L. paracasei* subsp. *paracasei*, and showed greater than 75% RAPD profile similarity to *L. casei* LMG 6904 (see Chapter 2).

5.4.2. GROWTH KINETICS

Diversity in *L. acidophilus* growth characteristics was observed, and differences were measureable at a statistically significant level. The most marked differences between groups were seen between *L. acidophilus* isolates and the non- *L. acidophilus* out-group. This was particularly exemplified by the greater culture density reached by the non- *L. acidophilus* group, which was significantly higher than all other groups except the MMS3 group containing isolates exposed to MMS three times. The ability of an isolate to grow to high densities is presumably a useful trait from a commercial probiotic perspective, particularly in the case of large-scale cultures that are freeze-dried and added into dairy products, but in capsules. Within the *L. acidophilus* groups, no significant differences in any growth characteristics were observed between the culture collection and commercial groups, which were assigned to isolates based on the diversity of their genome sequences (see Chapter 4). This indicates that the small differences observed in gene sequence and content between these groups isolates do not have a significant impact on their growth characteristics. It should be noted however, that translating these effects from the microculture discussed in this chapter to the large-scale cultures required for industrial production of probiotic products should be done with caution, as just a small increase in the size of the culture vessel may have profound effects on the growth characteristics of a particular strain (Arnold et al., 1999).

The groups containing isolates that were exposed to MMS once, twice or three times (MMS1, MMS2 and MMS3 respectively) were originally derived from *L. acidophilus* NCFM, a member of the commercial *L. acidophilus* group. Drawing comparisons between the MMS exposed isolates and the commercial *L. acidophilus* group showed no significant difference in overall culture density reached. The MMS1 group had a weakly ($p<0.05$) significantly increased growth compared to the commercial *L. acidophilus* group, but no other growth-rate effects were observed. The effect of MMS exposure is most obviously observed when comparing the lag-times of exposed isolates to their parent culture collection isolates; all are significantly longer. The phenomenon of sub-lethal stress resulting in increased culture lag-times is well documented in *Lactobacillus* (Smelt et al., 2002) and other genera (Guillier et al., 2005), and it appears that exposure to MMS has induced the same response in *L. acidophilus* NCFM.

5.4.3. MALDI-TOF MS PROFILING OF *LACTOBACILLUS* ISOLATES

Several recommendations were made by the EU-PROSAFE project as areas for further research in the area of identification of LAB (Vankerckhoven et al., 2008). MALDI-TOF MS profile analysis was implicated as a highly desirable, rapid and cost-effective phenotypic method to augment and validate sequence-based taxonomy and identification schemes for commercial probiotic LAB cultures (Vankerckhoven et al., 2008). Automated systems using MALDI-TOF MS to identify unknown isolates are already commercially available (Bruker Daltonik GmbH, 2010), but identification by these systems is achieved by matching new MALDI-TOF profile entries with a database of known profiles and calculating similarity scores (Bruker Daltonik GmbH, 2010). The approach used in this chapter used only data generated by this study in a purely comparative way. As the characterisation of the isolates tested has been undertaken on multiple levels, from DNA fingerprinting (see Chapter 2) to whole genome sequence (see Chapter 4), this chapter describes a purely comparative study that illustrates the power of MALDI-TOF MS profile analysis to both accurately place similar *Lactobacillus* isolates close together, and disparate isolates further apart (Section 5.3.3; Figure 5.6). The developed workflow allowed visualisation and validation of data on multiple scales, from comparisons of single MALDI-TOF MS spectra, to comparisons of more than 100 *Lactobacillus* profiles. MALDI-TOF MS profile analysis has been shown to accurately resolve closely related LAB species in the genera *Leuconostoc* and *Pediococcus* (De Bruyne et al., 2011), and the data presented in this chapter suggest that it is a similar case for *Lactobacillus*. MALDI-TOF MS profile analysis also has the potential to resolve isolates at the sub-species level (Vargha et al., 2006), with a strain-level resolution comparative to that of MLST in certain studies (De Bruyne et al., 2011). The advantages of MALDI-TOF include that it does not require genomic DNA, removing the cultivation step before DNA extraction required for MLST; that it requires no previous knowledge of DNA sequence in order to design efficient PCR primers targeting housekeeping genes which is essential for MLST and that bacterial colonies may be analysed directly, enabling extremely fast typing of isolates.

When applied to *Lactobacillus* isolates from food products using the Bruker BioTyper system (Bruker Daltonik GmbH, 2010), MALDI-TOF MS and PCR-based identification concurred in 95.6% of isolates (Dušková et al., 2012). The approach taken in this chapter

was able to classify all *Lactobacillus* species tested into different profile types, with the exception of *L. gasseri* LMG 9203 and *L. johsonii* LMG 9436, both closely related members of the *L. acidophilus* phylogenetic subgroup. This again illustrates the value of a polyphasic approach to typing, as the additional information gathered from RAPD profile similarity analysis (see Chapter 2) allows the classification of these two isolates as two distinct taxa.

When considered as a single taxonomic entity, *L. acidophilus* isolates generated diverse MALDI-TOF profiles, although all isolates were more similar to other *L. acidophilus* isolates than any other *Lactobacillus* species, and no obvious divergent groups of *L. acidophilus* were observed (Figure 5.6). When divided into the groups devised by genomic analysis (see Chapter 4), the diversity of MALDI-TOF profiles both within and between culture collection and commercial isolates was approximately equal (Figure 5.7), i.e. the variation in MALDI-TOF profiles generated by *L. acidophilus* isolates was not associated with their genome sequence. Additionally, the most genomically homogenous group of *L. acidophilus* isolates, those from commercial probiotic products, generated a range of MALDI-TOF profiles similar in diversity to the more genomically diverse culture collection isolates (Figure 5.7). In a species that shows greater genomic conservation than *L. acidophilus*; *Bifidobacterium animalis* subsp. *lactis*, MALDI-TOF profiles were seen to vary by up to 4% (Ruiz-Moyano et al., 2012). This shows that there is at least some MALDI-TOF profile variation that cannot be explained by genetic diversity been isolates, and is more likely attributable to sample preparation and growth conditions (Liu et al., 2007).

5.4.4. SUMMARY AND PERSPECTIVE

The variation in phenotypic characteristics of *L. acidophilus* isolates observed in this chapter is typical of phenotypic approaches to characterising bacteria in general. The phenotype of a particular isolate may be influenced by a vast array of external factors, including but not limited to culture conditions (e.g. temperature and oxygen availability) stress and growth phase. This makes generating consistent results particularly difficult, and because of this, phenotypic approaches have been complemented and to a large extent

superseded by genotypic approaches (Stackebrandt et al., 2002). As identified by the EU-PROSAFE project (Vankerckhoven et al., 2008), the accurate identification of probiotic cultures is imperative, particularly if they are sold commercially. This chapter emphasises the requirement for a polyphasic approach to probiotic characterisation, and using both genotypic and phenotypic elements as part of that approach ensures a comprehensive description of a probiotic culture.

5.5. CONCLUSIONS

The main conclusions from this chapter are as follows:

5) Carbohydrate fermentation profiles of *L. acidophilus* isolates vary considerably. The API 50 CHL test was unable to classify well classified isolates based on carbohydrate fermentation ability alone.

6) The carbohydrate fermentation profile of *L. casei* (*L. acidophilus*) TBCC was markedly different to *L. acidophilus* profiles. The reclassification of this isolate based on RAPD profile analysis is supported by the API 50 CHL test.

7) There were no significant differences in maximum culture density, maximum growth rate or lag time between culture collection and commercial *L. acidophilus* isolates.

8) MMS exposed *L. acidophilus* isolates did not grow to different culture densities or with different maximum growth rates to their parent, commercial *L. acidophilus* group. The lag phase of all MMS exposed isolates was significantly longer than the commercial *L. acidophilus* group.

9) MALDI-TOF profile analysis was able to differentiate all tested Lactobacillus species, with the exception of *L. gasseri* and *L. johsonii*. *L. acidophilus* isolates showed diversity in their generated MALDI-TOF profiles which was not related to their genotypes.

6. COMPARATIVE GENOMICS OF COMMERCIAL *BIFIDOBACTERIUM* STRAINS

6.1. INTRODUCTION

The human GIT is host to a large, diverse and dynamic microbial community, of which *Bifidobacterium* is a dominant genus (Gill et al., 2006). The overall number and composition of bifidobacterial species in the human intestine is dynamic over time (Barrangou et al., 2009). From birth, particularly following vaginal delivery, the GIT of healthy neonates is colonised by bifidobacteria, especially in breast-fed infants during early life (Jost et al., 2012). Variation in the composition of the gut microbiota between individuals however, is remarkable in infants (Jost et al., 2012), and this is reflected in the variation of dominant genera seen in different metagenomic studies of the human gut microbiota (Gill et al., 2006, Palmer et al., 2007, Ley et al., 2008). The microbiome of the infant GIT becomes more diverse as the diet encompasses greater complexity, although *Bifidobacterium* is typically the dominant genus until weaning (Jost et al., 2012).

Bifidobacteria are also intensively used in functional foods, as they may exert a health benefit on their human host (see Chapter 1). The causal molecular mechanisms for these health-promoting activities however, are still relatively uncharacterised. Recently, the sequencing and analysis of genome sequences of probiotic bacteria, specifically aimed at the discovery of genetic determinants responsible for the adaptation to the gastrointestinal tract of their host, has been referred to as "probiogenomics" (Ventura et al., 2012). Combined with *in vivo* and *in vitro* characterisation of bacterial-host interactions, probiogenomics offers a powerful means to elucidate the molecular mechanisms behind beneficial probiotic effects.

Bifidobacterium animalis subsp. *lactis* is a Gram-positive lactic acid bacterium commonly found in the GIT of healthy humans (Turroni et al., 2009). From a probiotic perspective, the benefits associated with strains of *B. animalis* subsp. *lactis* have resulted in their

inclusion in the human diet via a large array of dietary supplements and foods, including dairy products such as yoghurts (Barrangou et al., 2009). As a result, *B. animalis* subsp. *lactis* has become the most common bifidobacterium species utilised as a probiotic in commercial dairy products in North America and Europe (Gueimonde et al., 2004). Despite this extensive commercial and probiotic importance, the strain-level differentiation of *B. animalis* subsp. *lactis* using classical genotyping techniques such as PFGE (Section 1.5.1.1) has been hindered by the high genetic similarity of these organisms. Fortunately, the availability of genome sequences for nine *B. animalis* subsp. *lactis* isolates (Table 1.6) allows an unprecedented ability to identify the genetic and pan-genome variation within this species and in comparison to other members of the genus. From examination of these genomes, the extent of genetic variability occurring among members of *B. animalis* subsp. *lactis* has been shown to be remarkably low (Milani et al., 2013).

Studies of the ecology of *Bifidobacterium bifidum* show a similar niche to *B. animalis* subsp. *lactis,* as another dominant species within the infant GIT microbiota (Turroni et al., 2009). In contrast to *B. animalis* subsp. *lactis*, gross genomic statistics relating to draft and complete genome sequences of *B. bifidum* strains show that genome size, GC (%) content and number of ORFs are variable between strains. *B. bifidum* strains have also seen use in multiple probiotic and food supplements (Shah et al., 1995, Masco et al., 2005), including the Lab4® formulation used by Cultech Ltd. As a final component of this PhD study, phylogenomic analysis of *B. bifidum* and *B. animalis* subsp. *lactis* isolates was conducted to examine the genomic similarity and phylogenetic placement of two commercial *Bifidobacterium* isolates, *B. bifidum* CUL 20 and *B. animalis* subsp. *lactis* CUL 34, used as part of the Lab4® probiotic produced by Cultech Ltd.

6.1.1. SPECIFIC AIMS

The aims of this chapter were as follows:

- Obtain draft genome sequences for two commercial *Bifidobacterium* isolates, *Bifidobacterium bifidum* CUL 20 and *Bifidobacterium animalis* subsp. *lactis* CUL 34

- Use comparative genomics to establish the evolutionary history and phylogenetic placement of commercial isolates

- Develop molecular markers for detecting *Bifidobacterium bifidum* at the species level

- Test molecular markers in culture independent approach to establish their efficacy and investigate distribution of bifidobacteria within the GIT.

6.2. METHODS

6.2.1. COMMERCIAL *BIFIDOBACTERIUM* GENOME RESEQUENCING

As previously (see Section 4.2.1.2), high molecular weight genomic DNA was extracted from the growth of single-colony overnight cultures (see Section 2.2.2) with a Wizard genomic DNA purification kit (Promega, Southampton, United Kingdom), following the manufacturer's instructions. Genomic DNA libraries were prepared and single-reads were sequenced by GATC Biotech (Konstanz, Germany) using an Illumina HiSeq2000 with 50 bp sequence read length. Genome sequences were assembled using Velvet version 1.2.01 shuffle and optimisation scripts, creating contigs with optimal parameters, with *k*-mer lengths between 21 and 51 bp (Table 6.1) (Zerbino, 2010, Cody et al., 2013). Assembled data were deposited in the PubMLST database as implemented by the Bacterial Isolate Genome Sequence Database (BIGSDB) software platform (Jolley and Maiden, 2010). To supplement and contextualise the new sequence information generated by this study, further completed and draft genome sequence data from strains of *B. bifidum*, *B. animalis* subsp. *lactis* and other bifidobacteria were downloaded from the Integrated Microbial Genomes (IMG) database (Markowitz et al., 2010) and also deposited into the PubMLST database (Table 6.2).

6.2.2. GENOMIC DIVERSITY ANALYSIS OF BIFIDOBACTERIA USING RMLST

Relationships between *Bifidobacterium* genomes, from genus- to strain-level, were identified using phylogenetic networks based on rMLST sequences. The 53 ribosomal subunit loci identified in the automated annotation process (described in Section 4.2.2) were compared among all isolates using the BIGSDB Genome Comparator module. The distance matrix generated on the basis of shared alleles was visualized with the Neighbor-Net algorithm (Bryant and Moulton, 2004), implemented in SplitsTree version 4.8 (Huson and Bryant, 2006) within the BIGSDB Web-interface (Jolley and Maiden, 2010).

6.2.3. GENOMIC DIVERSITY ANALYSIS OF *B. BIFIDUM* AND *B. ANIMALIS* SUBSP. *LACTIS* STRAINS USING "GENOME-WIDE" MLST

Genome sequences of *B. bifidum* and *B. animalis* subsp. *lactis* isolates were analysed at the strain level using BIGSDB Genome Comparator. The genome sequences of *B. animalis* subsp. *lactis* strains were compared at all loci defined in the genome sequence of the type strain DSM 10140 (GenBank accession: CP001606). For the *B. bifidum* genomes loci comparison was made against those defined in *B. bifidum* BGN4 (GenBank accession: CP001361) because it was the genome sequence with the largest number of defined loci currently available. Presence/absence of each locus in all genomes was established and sequence variation indexed at each locus, using the approach described in Chapter 4.

6.2.4. DEVELOPMENT OF SPECIFIC MARKERS FOR *B. BIFIDUM*

Regions of the *B. bifidum* genome that were unique to this species were found using INSIGNIA (Phillippy et al., 2009). Their distribution in all *B. bifidum* genomes including *B. bifidum* CUL 20 was confirmed using the BlastN function implemented by the BIGSDB (Jolley and Maiden, 2010). PCR primers targeting a selected unique region (ORF BBB_0726; see results) were designed using Primer3 (Table 6.3) (Rozen and Skaletsky, 2000). A second set of nested PCR primers was designed in the same way to improve on the sensitivity of the PCR (see Section 2.2.6). The specific marker PCR was tested on total faecal DNA extracted from the faeces of 10 healthy human adult males taking part in a Lab4® probiotic feeding trial (see Section 2.3.3.2). DNA samples tested corresponded to pre-feeding time points when the individuals were not taking probiotic containing *B. bifidum* CUL20 and 3 additional probiotic bacteria (see Chapter 1).

A nested PCR test for the presence of the region specific to *B. bifidum* was carried out as follows. A 25 µl PCR mixture was set up as follows: 1x PCR buffer, 1x Q-solution, 100 µM final concentration of dNTPs, 0.4 µM final concentration of each primer, 2 µl of template DNA (extracted from total faecal DNA (Sections 2.2.10 and 2.2.11) or from the first round of PCR), and 1 U of *Taq* DNA polymerase. The nested PCR was carried out in two stages using a BioRad C1000 thermal cycler (BioRad, Hemel Hempstead, United Kingdom) the first using the program; 94°C for 180 sec, then 30 cycles of 30 sec at 94ºC, 2

min at 59ºC, 60 sec at 72ºC, then a final step at 72ºC for 10 min. The product from this PCR was then used as the template DNA for the second round of PCR using the same program. The resulting PCR products were visualised by agarose gel electrophoresis on a 1.5% (w/v) gel.

Table 6.1: Commercial *Bifidobacterium* isolates resequenced as part of this study

Species	Strain	Number of sequence reads	Read Length	Contig N50	Number of contigs >1kb
Bifidobacterium animalis subsp. *lactis*	CUL 34	16792017	51	50662	60
Bifidobacterium bifidum	CUL 20	23278433	51	56725	85

Table 6.2: *Bifidobacterium* genome sequences used in this study

Species	Strain	Source	Genome status	rMLST type
Bifidobacterium animalis subsp. *lactis*	Bl-04	human fecal	finished	1
Bifidobacterium animalis subsp. *lactis*	DSM 10140	yogurt	finished	2
Bifidobacterium animalis subsp. *lactis*	HN019		draft	1
Bifidobacterium animalis subsp. *lactis*	BB-12		finished	1
Bifidobacterium animalis subsp. *lactis*	V9	human faeces	finished	1
Bifidobacterium animalis subsp. *lactis*	B420		finished	1
Bifidobacterium animalis subsp. *lactis*	Bi-07		finished	1
Bifidobacterium animalis* subsp. *lactis	**CUL 34**	**Cultech**	**Draft; this study**	**1**
Bifidobacterium bifidum	NCIMB 41171	human	Draft	3
Bifidobacterium bifidum	IPLA 20015		Draft	4
Bifidobacterium bifidum	LMG 13195		Draft	5
Bifidobacterium bifidum	S17		Finished	6
Bifidobacterium bifidum	PRL2010		Finished	7
Bifidobacterium bifidum	BGN4	human feces	Finished	8
Bifidobacterium bifidum	**CUL 20**	**Cultech**	**Draft; this study**	**9**
Bifidobacterium breve	DSM 20213	human	Draft	10
Bifidobacterium catenulatum	DSM 16992	human	Draft	11
Bifidobacterium dentium	ATCC 27678	human	Draft	12
Bifidobacterium gallicum	DSM 20093	human	Draft	13
Bifidobacterium longum	DJO10A	human	Finished	14
Bifidobacterium longum	NCC2705	infant faecal	Finished	15
Bifidobacterium pseudocatenulatum	DSM 20438	human	Draft	16

Genome sequences generated in this study are indicated in bold text

Table 6.3: PCR primers for *B. bifidum* ORF BBB_0726 specific marker region

PCR Primer	Melting temp	PCR Primer Sequence	PCR product size (bp)
Bbif_F	60.14	GTGACGATTGCGATACGTTG	413
Bbif_F	59.83	CAGCGGTAATAGCTCGATCC	
Bbif_NEST_F	60.16	CGATGAAACCGGATATGACC	106
Bbif_NEST_R	59.99	TCGAGCACCTGACTGATGAC	

6.3. RESULTS

This results section is divided into three sections as follows. The first applied comparative genomic methods, to examine genus and strain level diversity of *Bifidobacterium* using rMLST. The second applied intraspecies, genome-wide comparisons of *B. bifidum* and *B. animalis* subsp. *lactis* genomes, respectively, to examine the diversity of each species at all protein coding loci as defined by a reference genome sequence. The third evaluated a PCR test for the presence of *B. bifidum* using a specific marker sequence identified via a comparative genomics approach (Phillippy et al., 2007, Phillippy et al., 2009).

6.3.1. APPLICATION OF RMLST TO *BIFIDOBACTERIUM* GENOMES

rMLST was applied to the genome sequences of eight bifidobacterial species, including two isolates resequenced as part of this study; *B. animalis subsp. lactis* CUL34 and *B. bifidum* CUL 20. A Neighbour-net generated from the concatenated sequences of 52 *rps* protein coding loci placed *B. bifidum* CUL 20 within a diverse group of other *B. bifidum* isolates (Figure 6.1). In contrast, the *B. animalis subsp. lactis* CUL 34 strains was located within a group of highly related *B. animalis subsp. lactis* genomes, which were not clearly resolved by Neighbour-net analysis (Figure 6.1). Correlating to this observation, each *B. bifidum* genome tested had a unique rMLST profile (rMLST type 3 – 9; Table 6.2), while all rMLST profiles generated for *B. animalis* subsp. *lactis* isolates were identical (sequence type 1; Table 6.2), with the exception of *B. animalis* subsp. *lactis* DSM 10140, which differed by a single nucleotide at a single locus resulting in a different overall rMLST type (rMLST type 2; Table 6.2).

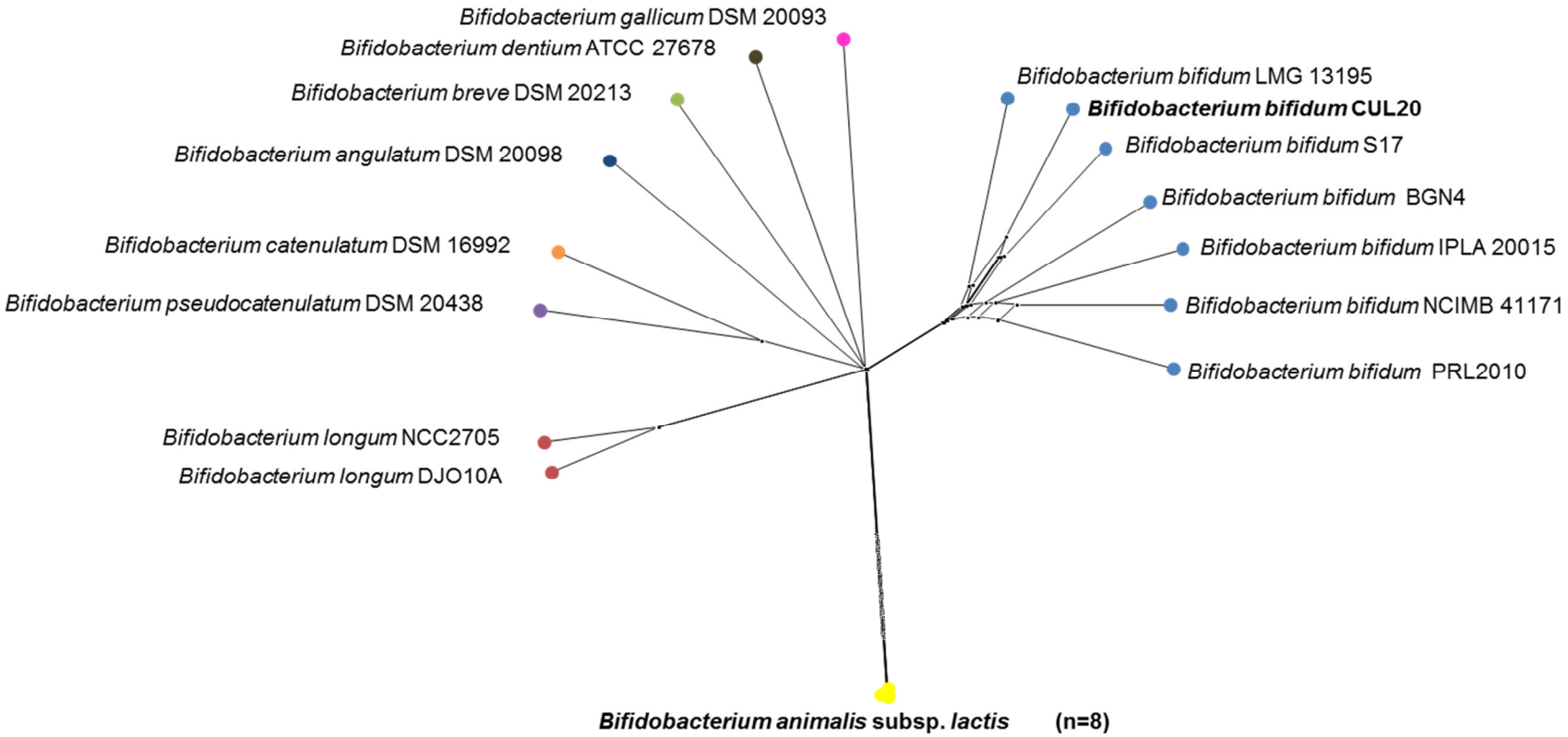


Figure 6.1: *Bifidobacterium* genus rMLST Neighbour-net

The analysis was based on the concatenated sequences of 53 ribosomal protein coding loci. An overall fit of 99.99 % indicated that this network accurately represented the original sequence data. Nodes are coloured by species and Cultech isolates are labelled in bold text.

6.3.2. GENOME-WIDE MLST

As rMLST was unable to differentiate *B. animalis* subsp. *lactis* isolates, genome-wide MLST (Section 4.2.3.1) was used to compare genome sequences of eight isolates. When searched for protein coding loci defined in genome sequence of the *B. animalis* subsp. *lactis* type strain, DSM 10140, of 1560 defined loci 1554 (99.6%) were found in all *B. animalis* subsp. *lactis* genomes tested (Figure 6.2). Of the remaining six protein coding loci, five were absent only from *B. animalis* subsp. *lactis* HN019 and a single protein coding locus absent from *B. animalis* subsp. *lactis* CUL 34 (Table 6.4). When examined at the sequence level in all *B. animalis* subsp. *lactis* isolates, just 58 loci were found to have coding sequences with internal nucleotide diversity. 1470 (94%) loci had identical sequences in all isolates, while 26 loci were found to have identical sequences in all isolates apart from *B. animalis* subsp. *lactis* DSM 10140.

In contrast, *B. bifidum* was considerably more diverse when all protein coding loci corresponding to those defined in the genome sequence of *B. bifidum* BGN4 were examined. The mean *B. bifidum* genome size was larger (2.2 Mb ± SEM = 0.014) than that of *B. animalis* subsp. *lactis* genome (1.94 ± SEM = 0.002), and had 17% more protein coding loci defined within its genome sequence. Of the 1835 protein coding loci defined in the *B. bifidum* BGN4 genome sequence, 1565 (85%) were present in all other *B. bifidum* genome sequences analysed (Figure 6.2). 18 (<1%) protein coding loci defined in the genome sequence of *B. bifidum* BGN4 were identical in all other *B. bifidum* strains at the sequence level (Table 6.5). When the sequences of the shared loci of *B. bifidum* were analysed, there was no evidence of groups of genome sequences, each sequence was equally as distant from those within the other genome sequences.

Table 6.4: Loci absent from *B. animalis* subsp. *lactis HN019* and CUL 34

Locus	Product	Sequence length	Genome position	HN019	CUL 34
Balat 0510	50S ribosomal protein L31	210	604073	X	T
Balat 0523	50S ribosomal protein L31	213	622554	X	T
Balat 1572	ABC transporter solute-binding protein	1335	1854711	T	X
Balat 1582	NAD synthetase	537	1868120	X	-
Balat 1583	NAD+ synthetase	1335	1868691	X	-
Balat 1584	hypothetical protein	270	1870189	X	-

X indicates missing locus

T indicates truncated locus

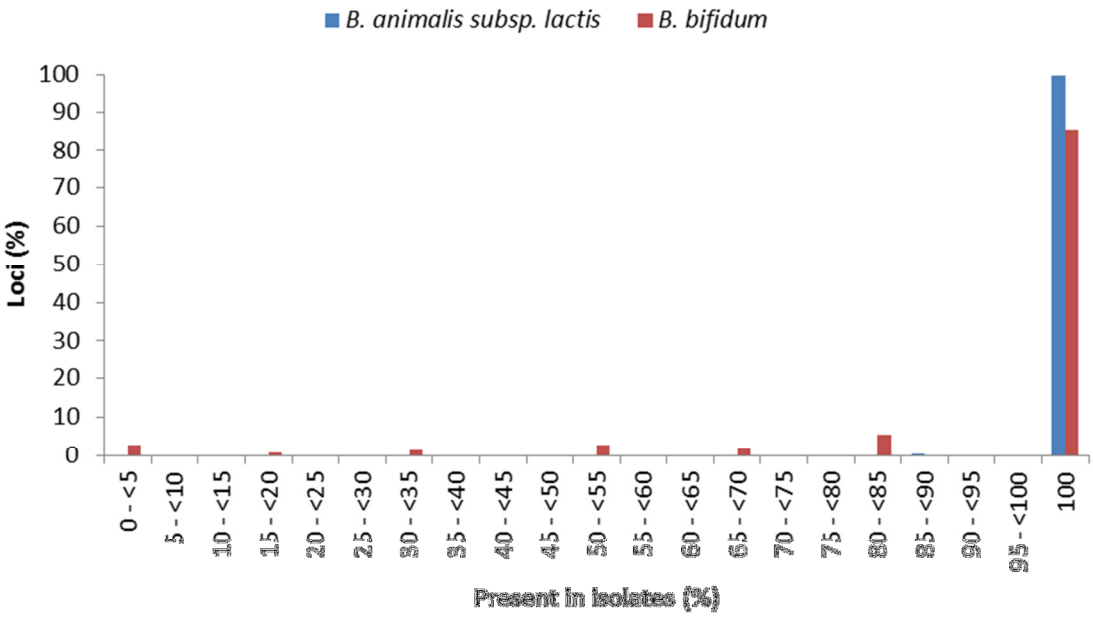


Figure 6.2: Percentage of protein coding loci found in *B. animalis* subsp. *lactis* strains and *B. bifidum* strains

Table 6.5: Protein coding loci with identical DNA sequence in all *B. bifidum* genomes

Locus	Product	Sequence length	Genome position
BBB 0216	hypothetical protein	126	263969
BBB 0298	ribosomal protein L28	195	358485
BBB 0337	hypothetical protein	252	403695
BBB 0386	hypothetical protein	273	464161
BBB 0565	hypothetical protein	69	698955
BBB 0619	DNA-binding protein	282	759720
BBB 0786	modification methylase	246	954966
BBB 0995	hypothetical protein	147	1195074
BBB 1290	hypothetical protein	219	1537073
BBB 1379	ribosomal protein L31	213	1648437
BBB 1431	conserved hypothetical transmembrane protein	132	1705113
BBB 1498	ribose 5-phosphate isomerase A	699	1793503
BBB 1499	hypothetical protein	90	1794369
BBB 1522	ribosomal protein L30	183	1822970
BBB 1527	30S ribosomal protein S14 type Z	186	1825301
BBB 1565	hypothetical protein	117	1859690
BBB 1674	aspartyl/glutamyl-tRNA (Asn/Gln) amido transferase subunit C	330	1996481
BBB 1842	ribosomal protein L34	135	2223178

6.3.3. DEVELOPMENT AND TESTING OF A PCR TEST FOR THE PRESENCE OF *B. BIFIDUM*

Using INSIGNIA, the single copy ORF defined as BBB_0726 which encoded a predicted phosphomannomutase within the genome sequence of *B. bifidum* BGN4 was selected as a likely unique marker for *B. bifidum*. The ORF had the following features: (i) 1176 bp in length; (ii) encoded a protein of 275 amino acids, with a predicted molecular mass of 29,937 Da; and (iv) encoded a phosphomannomutase Pfam domain predicted to be involved in the synthesis of GDP-mannose and dolichol-phosphate-mannose.

To establish the wider prevalence and conservation of ORF BBB_0726, its nucleotide sequence was used to probe all *Bifidobacterium* genome sequences available at the BIGSDB using BlastN (Table 6.6). Only *B. bifidum* genomes showed the presence of the complete sequence, producing, 1176 bp alignment lengths that were representative of conservation of across the entire gene sequence. The alignments were used to design specific PCR markers for *B. bifidum* specifically avoiding any mismatches; similarly, nested PCR primers were designed to internal conserved regions of BBB_0726 to allow potential detection of low levels of *B. bifidum* DNA.

The *B. bifidum* specific markers were tested on total faecal DNA prepared from XX healthy male adults enrolled on a probiotic (Lab4®) feeding trial in a semi-quantitative manner, as no detection limits were defined for this test. After a first round of PCR, two participants tested positive for *B. bifidum* (Figure 6.3, panel a, lanes 1 and 6). After the second, nested round of PCR, all participants tested positive for the *B. bifidum* specific marker (Figure 6.3, panel b). PCR products were subsequently sequenced and confirmed to be identical in sequence to BBB_0726.

Table 6.6: Specific marker region in *Bifidobacterium* genomes

Species	Strain	% identity	Alignment length (bp)	Mismatches	Gaps
B. bifidum	NCIMB 41171	99.23	1076	9	0
B. bifidum	IPLA 20015	99.23	1076	9	0
B. bifidum	LMG 13195	99.40	1076	7	0
B. bifidum	S17	99.83	1076	2	0
B. bifidum	PRL2010	99.74	1076	3	0
B. bifidum	BGN4	98.89	1076	13	0
B. bifidum	CUL 20	100.00	1076	0	0
B. longum	DJO10A	75.79	318	65	11
B. longum	NCC2705	75.79	318	65	11
B. longum	ATCC 55813	75.79	318	65	11
B. longum	CCUG 52486	75.79	318	65	11
B. longum	ATCC 15697	75.47	318	66	11
B. breve	DSM 20213	75.82	273	60	5
B. dentium	Bd1	77.16	232	51	2
B. dentium	ATCC 27678	77.16	232	51	2
B. catenulatum	DSM 16992	76.45	242	50	7
B. animalis subsp. *lactis*	HN019	76.02	246	52	7
B. animalis subsp. *lactis*	CUL 34	76.02	246	52	7
B. animalis subsp. *lactis*	Bl-04	76.02	246	52	7
B. animalis subsp. *lactis*	DSM 10140	76.02	246	52	7
B. animalis subsp. *lactis*	AD011	76.02	246	52	7
B. animalis subsp. *lactis*	BB-12	76.02	246	52	7
B. animalis subsp. *lactis*	V9	76.02	246	52	7
B. animalis subsp. *lactis*	B420	76.02	246	52	7
B. animalis subsp. *lactis*	Bi-07	76.02	246	52	7
B. angulatum	DSM 20098	74.00	200	52	0
B. adolescentis	ATCC 15703	73.31	236	51	8
B. pseudocatenulatum	DSM 20438	73.37	199	53	0
B. gallicum	DSM 20093	92.16	51	4	0
B. adolescentis	L2-32	72.77	235	54	9

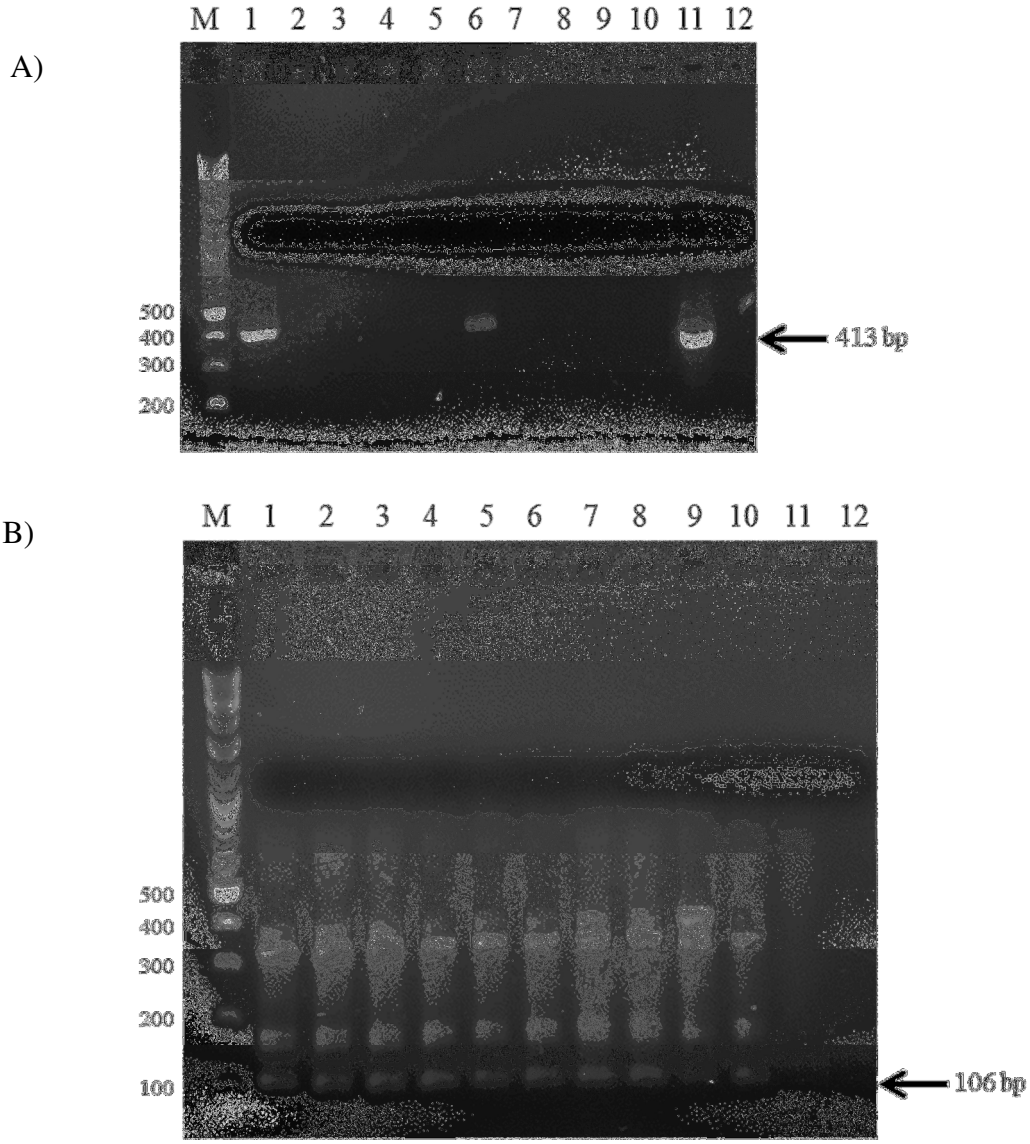


Figure 6.3: Examining adult total faecal DNA from healthy human males enrolled on a probiotic (Lab4®) feeding trial (pre-feeding), for the presence of *B. bifidum*

Panel (A) shows PCR products from 10 total faecal DNAs (lanes 1-10), a negative PCR water control (lane 12) and *B. bifidum* CUL 20 DNA as a positive control (lane 11). Panel (B) shows the same, with DNA testing positive for *B. bifidum* DNA in panel (A) removed. An additional *B. bifidum* CUL 20 DNA positive control for the second round of PCR (lane 10) and an additional water PCR control for the second round of PCR lane 12). The 413 bp (panel A) and 106 bp (panel B) predicted PCR product sizes (Table 6.3) are indicated. Lane M contains molecular size marker with sizes of relevant bands given in bp.

6.4. DISCUSSION

Overall, the genus *Bifidobacterium* encompasses a large amount of genomic diversity and a wide pan-genome, with few functions shared across all species. In fact, the gene pool pertaining to the whole genus is more than twice the size of a single bifidobacterial genome, and the pan-genome of the genus is described as open (Bottacini et al., 2010). Within bifidobacterial species however, the story is very different. Genomic diversity exists, but individual species are more likely to form discreet pan-genomic units within themselves (Bottacini et al., 2010). Here we indexed the genomic diversity in two different bifidobacterial species, *B. animalis* subsp. *lactis* and *B. bifidum,* and found the possessed differing levels of intraspecies genomic identity. This was most clearly reflected when the number of genomic loci that share identical sequences across all isolates of a species was examined; 96% of loci examined were identical in sequence among the in *B. animalis* subsp. *lactis* genomes, contrasting with less than 1% of loci sharing the same sequence in the *B. bifidum* genomes examined.

6.4.1. APPLICATION OF RMLST TO BIFIDOBACTERIAL STRAINS

The diversity of *B. animalis* subsp. *lactis* and *B. bifidum* genomes was well reflected by the rMLST analysis (Figure 6.1). The Neighbour-net showed the high level of *rps* gene sequence conservation in all *B. animalis* subsp. *lactis* genomes, which was also echoed when observed at the genome-wide level. In contrast *B. bifidum*, exhibited much greater diversity in *rps* gene sequence, mirrored by its more diverse genome-wide MLST profile, with conserved sequences detected across all genomes for just 18 loci. rMLST is a useful tool therefore, for reflecting genomic diversity of bifidobacteria using a limited set of phylogenetic markers that describes genome evolution across a diverse range of *Bifidobacterium* isolates, without the need to investigate phylogeny based on an entire core-genome as in Bottacini *et al* (2010).

6.4.2. GENOMIC DIVERSITY OF *B. ANIMALIS* SUBSP. *LACTIS* STRAINS

It is clear that *B. animalis* subsp. *lactis* isolates have highly conserved genome sequences, with all isolates for which genomes are available being highly congruent in sequence. Previous research has shown that for 10 *B. animalis* subsp. *lactis* isolates that were examined, shared more than 99.82% sequence identity across their entire genome sequences and possessed 1,518 identical ORFs (Milani et al., 2013). Milani *et al* (2013) used a manual annotation approach to assign protein coding regions to each genome individually, and subsequently compared predicted proteomes of strains to find that only 3 ORFs were variable in presence among the isolates examined although their existence was not experimentally validated. This highlights the variability inherent in annotating genome sequences, particularly when multiple annotation platforms are used.

The genome-wide MLST approach does not suffer this pitfall, as it only requires a single reference annotation set to which other genome sequences are compared, either as contigs or as a complete genome sequence. Here we found no evidence of the 3 variable ORFS encoded in any *B. animalis* subsp. *lactis* genomes in comparison to the Milani *et al.* (2013) study. When examined for the presence of all protein coding loci defined in the type strain *B. animalis* subsp. *lactis* DSM10140, just six loci were found to be variably present, and with the entire set missing from *B. animalis* subsp. *lactis* HN019 and a single ORF, Balat 1572 missing from CUL34, the only draft genome sequences included in this analysis (Table 6.4). The truncation of two loci; Balat_0510 and Balat_0523, in *B. animalis* subsp. *lactis* suggests that they are present, but at the border of a contig. Both ORFs are identical in sequence in *B. animalis* subsp. *lactis* suggesting that the truncation occurred as a result of an assembly fault. The draft genome of *B. animalis* subsp. *lactis* HN019 is smaller (1916 kb) than the complete genomes of all other *B. animalis* subsp. *lactis* isolates (1938 kb), so it was inevitable that sequence data would be seen as absent in the comparative analysis. To confirm the true absence of these ORFs, experimental validation via amplification and sequencing of this genomic region would be required.

The addition of the draft genome sequence data generated from *B. animalis* subsp. *lactis* CUL34 did not alter hypothesis that *B. animalis* subsp. *lactis* is a highly clonal taxon with its genomic content encoding markedly similar exoproteomes. The reason for the highly isogenic nature of this species may be a recent divergence from *B. animalis* subsp. *animalis* by reductive adaptation to growth in yoghurt (Lee and O'Sullivan, 2010). *B. animalis* subsp. *lactis* genomes are unique within bifidobacteria because they have no genes, or an incomplete number, predicted to be involved in the utilisation of complex carbohydrate and polyols such as arabinofuran, arabinogalactan, arabinan, cyclodextrin, xylan and sugar alcohols (Lee and O'Sullivan, 2010). The ability to utilise complex carbohydrates is a common feature of GIT adapted bacteria, and the prolonged exposure of bifidobacteria to environmental niches with less complex nutrient availability has been shown to cause deletion of superfluous genomic loci (Lee et al., 2008). The genome reduction evolutionary step from *B. animalis* subsp. *animalis* to *B. animalis* subsp. *lactis* may also have implications for its probiotic ability, as bifidobacteria with reduced genomes have been shown to be less competitive than their progenitor strains with larger genomes (Lee et al., 2008).

6.4.3. GENOMIC DIVERSITY OF *B. BIFIDUM* STRAINS

In direct contrast to *B. animalis* subsp. *lactis, B. bifidum* genomes are considerably more diverse in both presence and absence of protein coding loci, as well as the nucleotide sequences of loci that are present. *B. bifidum* genomes shared fewer loci between one another than *B. animalis* subsp. *lactis* genomes, as evidenced by a lower percentage of loci present in 100% of genomes, and increased numbers of loci present in just one or more genomes (Figure 6.2). The loci that are conserved in sequence in all *B. bifidum* strains commonly encode proteins that are related to the ribosome (Table 6.5), whose sequence is would be expected to be highly conserved within species (Yutin et al., 2012). Another frequently encountered feature of *B. bifidum* genomics reflected in this analysis (Table 6.4) was the large numbers of ORFs of unknown function. It has been hypothesised that the proteins encoded by these hypothetical loci may play important roles in the interaction of bifidobacteria with the human host (Gueimonde et al., 2012). Further research will be needed to define these exact functions however. Previous comparative genomic analysis of eight *B. bifidum* strains indicated that there were large regions of the genome that vary both

in presence and sequence among the strains analysed (Turroni et al., 2010), corroborating the findings of our own study.

6.4.4. DEVELOPMENT AND TESTING OF A *B. BIFIDUM* SPECIFIC MARKER

A simple species-specific PCR test for *B. bifidum* based on conservation of the species-unique BBB_0726 ORF was developed (Table 6.3). In this study, access to a limited number bifidobacterial isolates or defined strains meant that the specificity of the region was tested *in silico* using tools at the BIGSDB (Section 6.4.4), and when the NCBI non-redundant database is search using BlastN, only sequences matching *B. bifidum* genomes are returned. BBB_0726 is predicted to encode a phosphomannomutase, an isomerase that catalyses the structural rearrangement between alpha-D-mannose 1-phosphate and D-mannose 6-phosphate. It is a carbohydrate-modifying enzyme that is part of the glycosylation pathway, which is well developed in *Bifidobacterium* species (Barrangou et al., 2009, Kim et al., 2009, Schell et al., 2002). The sequence of BBB_0726 was greater than 98% conserved in *B. bifidum* and was much more divergent in sequence in other bifidobacteria (Table 6.6), making it an excellent candidate for further development and evaluation as a species-specific marker gene.

The *B. bifidum* BBB_0276 marker showed in a semi-quantitative manner that 20% of healthy individuals tested were carrying *B. bifidum* at the higher detection limit, and the remaining 80% carried *B. bifidum* at levels higher than the lower detection limit, which in a similar test in L. acidophilus correlate to approximately 50 cfu and 5,000 cfu respectively. Bifidobacteria are found in relatively high numbers (up to 37% of total 16S rRNA gene sequences) in the human gut (Hill et al., 2010a), particularly in infants (up to 80% of total 16S rRNA gene sequences) (Turroni et al., 2009). Culture-independent studies using the 16S rRNA gene as a broad phylogenetic marker do not accurately distinguish these bacteria to the species level, and as such there is little reliable quantitative information available on the exact distribution of *B. bifidum* in the human gut.

This limited understanding of bacterial species distribution is especially evidenced in culture-independent studies markers other than the 16S rRNA gene, for example the *groEL* gene, is used to establish diversity profiles of the faecal microbiota (Hill et al., 2010a). Hill *et al* (2010a) hypothesise that the use of 16S rRNA to speciate bacteria in metagenomic studies underrepresents bifidobacterial species by up to 35%. Specific markers for *B. bifidum* based on the 16S rRNA gene have been previously described (Matsuki et al., 1998, Matsuki et al., 2003). A PCR test based on the 16S rRNA gene is useful for identification of pure cultures and presence/absence of the target organism in a qualitative approach. The multi-copy nature of the 16S rRNA operon in *B. bifidum* (approximately two copies per chromosome (Candela et al., 2004)) however, means that its utility for enumeration of cells is surpassed by a probe targeting a single-copy gene, such as BBB_0726. Further study to evaluate the detection limit of this test and additional development of a fully quantitative qRT-PCR test for *B. bifidum* based on BBB_0726 could be an excellent way to accurately quantify levels of *B. bifidum* in faecal material, especially when applied in a probiotic feeding trial.

6.4.5. SUMMARY AND IMPLICATIONS FOR COMMERCIAL *BIFIDOBACTERIUM* ISOLATES

The comparative genomic approach taken in this study corroborates existing information concerning the diversity of *B. animalis* subsp. *lactis*, namely that it is a clonal monophyletic taxon. Credible hypotheses for the highly clonal genome of this species have been presented and the addition of the genome sequence of *B. animalis* subsp. *lactis* CUL 34 follows the same pattern. *B. bifidum* is a more genomically diverse species, with the inclusion of the genome sequence of *B. bifidum* CUL 20 into analyses showing previously unseen sequences at some loci.

The highly clonal and reduced genomes of *B. animalis* subsp. *lactis* isolates means that data collected for a particular isolate can be extrapolated across the whole species with reasonable confidence. From a commercial perspective, this suggests that any claims made on probiotic products containing *B. animalis* subsp. *lactis* will be challenging to justify as specific to that particular product. The case for individual strains and distinct probiotic effects of *B. bifidum* is much more clear-cut, as although 85% of loci were shared between

all strains, their sequences were divergent and suggests they would encode unique probiotic activities at the strain level.

6.5. CONCLUSIONS

The conclusions from this chapter are as follows:

6. rMLST (*rps*) gene-based phylogenies are an effective and method for establishing both phylogenetic relationships and indexing genomic diversity within the genus *Bifidobacterium*.

7. Previous studies showing the clonality of *B. animalis* subsp. *lactis* isolates were corroborated with the genome-wide MLST approach. *B. animalis* subsp. *lactis* CUL 34 is a clonal member of this monophyletic taxon.

8. *B. bifidum* genome sequences were considerably more diverse, reflected in both rMLST and genome-wide MLST analyses. *B. bifidum* CUL 20 represents a novel strain with a genome sequence diverse from other isolates of *B. bifidum*.

9. The development of a *B. bifidum* specific PCR test, based on a single copy gene, is useful for detecting the presence of *B. bifidum* in total faecal DNA. Its specificity will require further testing, however if validated it could be further developed into a fully quantitative species-specific probe.

7. GENERAL CONCLUSIONS, DISCUSSION AND PERSPECTIVE

7.1.1. CONCLUSIONS

A comprehensive study describing the diversity of probiotic bacteria at the strain level was undertaken. Particularly in the case of *L. acidophilus*, a diversity analysis encompassing the number of isolates investigated in this study has never been undertaken. The techniques and methods applied reflected changing times in molecular strain-typing. Isolates of three major probiotic species were characterised and typed using classical phenotypic techniques, genotyped using conventional DNA fingerprinting techniques, which was then extended to the most novel, state-of-the-art whole-genome resequencing strategies. By using this range of methods we were able to index the variation between them at multiple levels, right down to the resolution of single nucleotide polymorphisms. The use of newly-developed next generation sequencing techniques also allowed the detailed examination of a hitherto unachievable number of genome sequences.

Below are the main conclusions from this study:

1. ***L. acidophilus*** **isolates show limited genetic diversity when assessed with RAPD. When their whole genome sequences are compared however, they separate into two broad groups representing commercial isolates and culture collection isolates, although the differences between the groups are small and the *L. acidophilus* core genome is stable across all isolates tested (Chapters 2 and 4).**

RAPD profile similarity analysis is an effective and highly reproducible method of examining their sub-species level genetic diversity. The *L. acidophilus* isolates we examined were from disparate isolation locations, deposition dates and host species. A single RAPD profile type, based on 75% profile similarity, was generated by all *L. acidophilus* isolates tested. This indicated that *L. acidophilus* isolates were all of a single strain type at the level of resolution

offered by RAPD analysis. Absolute genetic identity of these isolates could not be conclusively verified using RAPD profile similarity analysis alone, so further investigation at a discriminatory power beyond that of RAPD, was undertaken examine the underlying genetic structure of *L. acidophilus* and whether, as a species, it truly is clonally monophyletic.

Further to an initial survey with RAPD profile similarity analysis, a whole genome resequencing and comparison strategy was undertaken on a large collection of *L. acidophilus* isolates. This provided insight into the level of genomic variation required to translate to a significantly different RAPD profile type. *L. acidophilus* isolates broadly divided into two groups, representing isolates from culture collections and commercial isolates sold as probiotic supplements or cultivated from products. Limited RAPD profile variation had been observed within *L. acidophilus* isolates, but this did not correlate to the commercial or culture collection heritage of the isolates tested.

Commercial use or marketing a particular strain of *L. acidophilus* should be dependent on a unique phenotypic characteristic in relation to is probiotic or food-additive properties. Given the extensive homogeneity of the genome sequences of commercial isolates of *L. acidophilus,* our results potentially provides companies with the opportunity to assume that evidence of probiotic effect demonstrated in studies undertaken on one particular isolate of *L. acidophilus* would support their own strains equally as well. The demonstration of unique or enhanced probiotic ability in a single isolate may however set it apart from the others, but the validity of such a property may be questionable until detailed phenotypic studies encompassing the diversity of isolates seen in this study are conducted.

With very minor phenotypic differences among the *L. acidophilus* isolates observed in growth rate and protein-profile, it is hard to imagine given the level of clonal identity and limited number of non-synonymous mutations that any phenotypes of the isolates will differ greatly. This leads to hypothesis that more than any other factor, the commercial success of *L. acidophilus* lies in its stability and possession of phenotypes that have remained unaffected by

evolution due to mutation or horizontal gene transfer events. Perhaps like many other organisms it has been "domesticated" to be man's best friend when it comes to the microbial enhancement it brings to dairy products and digestive supplements used by humans over the ages.

2. *L. acidophilus* is a minor constituent of the GIT microbiota that is variably present in humans and was not detected in wild rodents (Chapter 2).

To attempt to ensure that the true genetic diversity of *L. acidophilus* was reflected by the isolates examined and to reduce the bias of our collection toward commercial and culture collection isolates, wild rodent faeces was examined as potential source of natural *L. acidophilus* isolates. To effectively search for *L. acidophilus* in wild rodent faeces, a species-specific molecular marker for *L. acidophilus* was developed and proved highly effective for detecting this species. No *L. acidophilus* was cultivated from wild rodent faeces or detected via the cultivation-independent approach, indicating that was either absent or present in numbers too low to detect within rodent faeces. Overall, our survey of wild rodents suggests that they do not naturally carry *L. acidophilus* within their GIT tract. Wider surveys of animal species using these tools may now be carried out to identify potential sources organisms beyond man and domesticated animals.

Also in the course of this study, the distribution of *L. acidophilus* human GIT carriage was estimated on a small-scale in healthy adults, using the species specific PAU-region PCR test. As with the wild rodent study, these probes were applied to total DNA extracted from faeces as an indicator of GIT carriage. When estimated in this way, it was shown that *L. acidophilus* carriage was highly variable, with between 32% and 75% of individuals carrying *L. acidophilus* at a low level. Corresponding culture independent studies indicate that lactobacilli may compose just 0.2-1% of the total microbiota in the human colon and faeces and also show that their prevalence is highly variable between individuals (Kleerebezem and Vaughan, 2009, Walter, 2008). Metagenomic studies commonly use molecular markers to elucidate bacterial community structure, particularly the sequence of the bacterial 16S rRNA gene. As

was shown in Chapter 3, the 16S rRNA gene sequences of certain *Lactobacillus* isolates are more than 98% similar when assessed across their whole length (>1200 bp) and potentially more similar if just a partial 16S rRNA gene sequence is used. To fully capture the diversity of *Lactobacillus* an alternative, more diverse molecular marker should be employed in culture independent diversity studies.

The gene that encodes the universal 60-kDa chaperonin protein Hsp60 has been used to study the diversity of *Bifidobacterium* in the GIT (Hill et al., 2010b) and may be a good alternative to the 16S rRNA gene for profiling very diverse communities of bacteria that contain clusters of very closely related species, such as the lactobacilli (Blaiotta et al., 2008). As a corollary, the diversity of the gut microbiome may not be particularly well estimated by studies that use faecal samples as an endpoint for community profiling, although it is a non-invasive method. Overall, gut carriage of *L. acidophilus* appears highly variable and while the species is clearly capable surviving passage through the gastrointestinal tract of humans (Mahenthiralingam et al., 2009), it is questionable as to whether the gut is the optimal or ancestral niche of this species.

3. **Comparative genomics and genome (re)sequencing of probiotic bacteria will become a "gold standard" method for characterisation and typing of isolates, and it is imperative that the metadata attached to publically available genome sequence information should be scrutinised stringently to ensure accuracy before subsequent analysis (Chapters 3, 4 and 6).**

We showed that the genome sequence of *L. acidophilus* 30SC was wrongly attributed to *L. acidophilus*, when it was more similar to *L. amylovorus* (Bull et al., 2012). The reliance of future analyses on the data provided with genome sequence depositions means that it is imperative that this metadata is correct. Fundamental components of this data are the correct taxonomic placement and species name that is attached to the genome record. The proliferation of genome resequencing means that a technology once restricted to the cutting-

edge of genomics is now available to a wider audience, including non-specialists and commercial parties. They may be unaware of such misidentification, and assume that a genome sequence deposited in a curated database and announced in a well-respected journal is to be trusted without performing their own analysis. Secondarily, organisms of considerable ecological, clinical and commercial interest, like *L. acidophilus* and *L. amylovorus,* may have probiotic effects that are strain-specific, and therefore ensuring that genome sequences of these bacteria are assigned the correct taxonomic nomenclature is vitally important. With a complete genome, researchers have all the information required to obtain the correct taxonomic nomenclature or evolutionary placement of a bacteria isolate.

To provide all interested parties, but particularly those that are not specialist genome (re)-sequencing laboratories, with a simple and effective method for establishing the provenance of their genome sequence, a bioinformatics workflow was developed. 16S rRNA, *gyrB* and *pheS* genes were designated as DNA biomarker genes and when applied to the *L. acidophilus* 30SC genome sequence the bioinformatics workflow showed that its genome was more similar to *L. amylovorus* LMG 9496. Secondarily, whole genome comparisons of available *L. acidophilus* and *L. amylovorus* genome sequences also showed that the genome of *L. acidophilus* 30SC showed greater synteny with *L. amylovorus* UCC1118 than *L. acidophilus* NCFM. This simple bioinformatic workflow and the need for correct genome taxonomy placement were recently validated by Mende *et al.* (2013), who developed a fully automated bioinformatic pipeline for bacteria species assignment using genome sequence data. Once it is certain that the data accompanying genome sequences are correct, the ability to integrate genome sequence data with a comparison platform, such as the BIGSDB, means that future phylogenetic analyses will be carried out using all of the information available within a genome sequence, attaching to it any pertinent phenotypic or physiological information that may be required for comparison. This type of analysis lends itself to investigating the evolution of particular physiological characteristics of bacterial strains and species.

4. **Methods for phenotypically characterising and typing LAB have generally been superseded in accuracy by DNA sequence based methods. Commercial and culture collection *L. acidophilus* isolates do not significantly differ phenotypically (Chapter 5).**

Conventional biochemical methods for identifying *L. acidophilus* such as carbohydrate fermentation profiling show that isolates of vary considerably in their phenotype. However, by their very nature, phenotyping tests like the API 50 CHL are less sensitive than typing tests based on nucleic acids, i.e. a single isolate may display a wide range of phenotypic test results and still be classified as the same species. The number of variable traits assessed by API 50 CHL is limited and cannot compare to either variation inherent in DNA sequence or potentially, the stability offered when the background genotype is stable and invariant as seen with *L. acidophilus.* This means that without any *a priori* knowledge of an isolate, API tests are demonstrably less accurate and reliable than DNA sequence based methods. They should no longer be used as a tool for commercial *L. acidophilus* quality control or typing unknown isolates, particularly when there are more accurate, faster and cheaper molecular methods to achieve the same result.

There were no detected significant differences in growth kinetics between culture collection and commercial *L. acidophilus* isolates. MMS exposed *L. acidophilus* isolates did not grow to different culture densities or with different maximum growth rates in comparison to their parental commercial *L. acidophilus* strain NCFM however, as a group, they did have a longer lag phase. *L. acidophilus* isolates showed limited diversity in MALDI-TOF profiles which was not related to their genotypes. Given that commercial *L. acidophilus* isolates were genomically homogenous, they did display a degree of phenotypic diversity when analysed with MALDI-TOF MS and API 50CHL carbohydrate profiling. As this diversity does not have an obvious foundation in the genome, it may be possible that exogenous circumstances such as culture conditions, stress, or the inherent differences in handling cells within these procedures leads to the minor phenotypic diversity seen in *L. acidophilus*. Potentially, if the proprietary status of commercial *L. acidophilus* isolates is lost because they are not demonstrably genomically different from company to company, the ability to manipulate and

obtain a stable and defining phenotype of probiotic *L. acidophilus* isolates could form new criteria from which to derive patents.

7.1.2. DELIVERABLE TOOLS AND STRATEGIES FOR TRANSLATION TO INDUSTRY (CULTECH)

7.1.2.1. Specific markers for *L. acidophilus* and *B. bifidum*

Species specific markers based on the PAU3 region in *L. acidophilus,* and a unique phosphomannomutase gene in *B. bifidum*, were developed as part of this study (Chapter 2 and Chapter 6). These molecular tools may be used to track these species as part of a culture-based feeding study, expanding on previous work which used RAPD profiles to type isolates (Mahenthiralingam et al., 2009), increasing throughput and ensuring a positive/negative test rather than one that relies on matching DNA fingerprints. When used in a culture independent context, the specific marker tests were designed as a two stage, nested PCR and hence provided a level of semi-quantitative information about the numbers of their respective target organisms. The single copy nature of the gene targets for these probes make them excellent candidates for development into a fully quantitative qRT-PCR test. This would allow future probiotic feeding studies to accurately enumerate numbers of *L. acidophilus* and *B. bifidum*, both before and after feeding, to examine the dynamics of the impact made on the gut microbiota in terms of probiotic numbers.

7.1.2.2. Development of a simple method for describing genome sequences before deposition

As previously discussed, the ever decreasing costs of high-throughput next-generation sequencing technologies means that genome (re)sequencing is becoming available to commercial enterprises and non-experts, instead of being restricted to a few, expert groups. Many commercial laboratories still use phenotypic techniques such as carbohydrate fermentation profiling to identify unknown isolates, and this data should not be relied upon alone to inform the metadata attached to a genome sequence. The developed bioinformatic workflow (Chapter 3) allows non-expert groups to confidently identify a genome sequence, and make a good estimate of its taxonomic placement. The genome sequence also provides an all-encompassing measure of quality control and stability of commercial strains and in future should be used as the gold standard measure for probiotic regulation and patent/commercial claims.

7.1.2.3. Draft genome sequences for all isolates within the Lab4® probiotic supplement

In 2008 (Vankerckhoven et al.) it was identified that conventional biochemical methods for characterising probiotic bacteria were insufficient and should be supplemented with molecular methods performed by an expert laboratory, such as those that maintain culture collections. With probiotic sales in the ascendancy, an important part of the marketing of probiotic products is the ability to label these foods with a health claim. Any petition to a regulatory agency for a health claim will have to name or describe the active ingredients in the product to be approved and provide evidence of efficacy (Farnworth, 2008). Polyphasic characterization combining phenotypic, biochemical, genotypic, and whole genome-sequencing results is considered to be the only way to reliably identify bacteria to the strain level (Mainville et al., 2006), which is important as even closely related bacterial species can have different probiotic properties. To obtain a health claim for a probiotic product, food manufacturers will have to precisely define their microorganism (Farnworth, 2008, Vankerckhoven et al., 2008). The availability of a set of almost-complete, draft genome sequences for the four probiotic strains marketed by Cultech, given in the context of other commercially available strains, provides invaluable information concerning their identity at a more complete level than can be achieved with all other existing methods such as RAPD, PFGE, carbohydrate utilisation profiling and MALDI-TOF MS.

7.1.3. GENERAL DISCUSSION

The implications and questions-raised during this study in the wider context of commercial probiotic usage and their history are discussed below. Focussed discussion of the results generated in each chapter is available at the end of each chapter and summarised here (see Section 7.1.1).

7.1.3.1. Why is *L. acidophilus* used as a probiotic?

The historical background for the use of *L. acidophilus* as a probiotic is well documented, but what was known as *L. acidophilus* in 1920 is vastly different to what is known as *L. acidophilus sensu stricto* in the present day (Figure 1.1). The elucidation of the probiotic functionality and physiology of *L. acidophilus* was undertaken after its status as a probiotic organism had been decided and "grandfathered-in" from the broader, historical definition of *L. acidophilus*. With the knowledge of the minor role of lactobacilli (*L. acidophilus* in particular) in the human GIT, at least in terms of it proportional representation, the question of why *L. acidophilus* is used as a probiotic is raised.

Historically, soured milk drinks were originally developed as preservation methods for milk products, and there are references to sour milk or fermented cultures as far back as the Bible (Anukam and Reid, 2007). Probiotic theory is attributed to Elie Metchnikoff who noted that the regular consumption of lactic acid bacteria in fermented dairy products, such as yoghurt, was associated with enhanced health and longevity in Bulgarian peasant populations. He linked this to the 'Bulgarian bacillus', a constituent of fermented milk products, which was discovered by a 27-year old Bulgarian physician Stamen Grigorov. Grigorov later had noted that bacteria in yogurt and other fermented milk products aided digestion and improved the immune system laying the foundations of potential probiotic health benefits (Grigoroff, 1905). Metchnikoff formalised this notion and posed the idea that aging was related to toxic bacteria in the gut. He proposed that if milk (kefir) soured with Bulgarian bacillus (now known as *L. bulgaricus* subsp. *bulgaricus*) was drunk regularly it mitigated the effects of aging and

"autointoxication" (Metchnikoff, 1907). A substantial number of studies were undertaken by contemporaries of Metchnikoff, investigating the effect of the application of *L. bulgaricus* subsp. *bulgaricus* to rabbits, guinea pigs and monkeys (Bested et al., 2013); preparations containing the bacterium were also made commercially available. Experimentation in 1920 showed that all attempts to colonise the GIT of humans with *L. bulgaricus* subsp. *bulgaricus* failed, whereas what was known as *L. acidophilus* at the time (Figure 1.1) was able to colonise successfully (Cheplin and Rettger, 1920). This led to the broad commercial adoption of *L. acidophilus* as a probiotic.

As a result of the commercial adoption of *L. acidophilus*, a great deal of its probiotic physiology has been documented *ex vivo*, under laboratory culture conditions and in isolation from the other microbiota that make up the highly complex and diverse gut. It therefore remains to be shown whether the same *ex vivo* probiotic physiologies are observed in the diverse and competitive GIT environment. Seemingly, the study of *L. acidophilus* in a probiotic context has been undertaken rather top down manner, deriving from the isolation of isolates capable of passage and apparent colonisation of the gut. What we now know to be a diverse group of lactose producing bacteria, originally used to preserve food whose consumption was postulated to prolong the life of early 20th century Bulgarians, were *assumed* to be probiotic. One could sceptically propose that the study of probiotic physiology markers for species "*assumed to be probiotic*" are often undertaken largely to support the conclusion that the particular species *is* probiotic. The high genomic and proteomic stability of *L. acidophilus sensu stricto* isolates observed in this study may go some way to explain the continued widespread commercial use and probiotic success it has. Such stability provides a guarantee that it can be grown and packaged reproducibly, remain unaffected by mutation/gene transfer/gene loss, and hence provide the same probiotic health effects over and over again. These are fundamental characteristics needed for commercial success of any product, and hence *L. acidophilus* can be likened to a stable currency for the probiotic market.

7.1.3.2. Commercial implications of clonal probiotics

It is highly likely that the global use of probiotic bacteria by man has influenced their genomic diversity, and ultimately even altered their population structure. This study shows that commercial probiotic isolates of *L. acidophilus* are genotypically more similar to themselves than isolates that have not been marketed as probiotic supplements. The homogeneity of genome sequences of commercial *L. acidophilus* isolates raises the question; what is the value of typing strains using molecular techniques such as RAPD, PFGE and MLST if appreciable sequence variation is not seen until the species level? The inability to distinguish one company's proprietary *L. acidophilus* isolate from another using methods such as PFGE, RAPD and MLST means that products will not satisfy the product label claim criteria set out by Farnworth (2008), unless *L. acidophilus* is treated as a clonally monophyletic species with no single proprietary isolates. Given the rapid ascent of next generation sequencing technologies, defining the genome of probiotic strains should now supersede the criteria set out by Farnworth *et al.* (2008)as a much more meaningful genotypic measure.

7.1.4. DIRECTIONS FOR FUTURE RESEARCH

1. **Establish the link between phenotype and genotype of *L. acidophilus* isolates. Even though the overall coding capacity of *L. acidophilus* genomes is very similar, does the difference in sequence within genes appreciably alter probiotic physiology?**

The effect of gene sequence on single probiotic physiological characteristics could be tested *ex vivo* using previously described assays that measure features such as tolerance to bile (Pfeiler et al., 2007) and low pH (Azcarate-Peril et al., 2004), ability to adhere to epithelial cells (Buck et al., 2005) and production of antimicrobial compounds (Tabasco et al., 2009). This would provide a demonstrable measure of how the probiotic effect differs in each particular isolate, and presumably would be useful for companies marketing *L. acidophilus* probiotics to differentiate their proprietary isolate from a competitor's. Moreover, given that we know the genetic basis for gene differences between the genome sequenced isolates, we are in a position to try to model exactly which pathways these minor differences would alter.

2. **Extend the semi-quantitative species specific molecular probes for *L. acidophilus* and *B. bifidum* into a fully quantitative test using quantitative PCR (qPCR).**

Highly specific, fully quantitative probes for probiotic species are useful in feeding trials. The function of a probiotic largely occurs in the large intestine, so therefore it must be established whether the probiotic reaches the large intestine, and in what numbers. Follow up validation of the PAU3 and *B. bifidum* PCRs using quantitative real time PCR approaches will be required to test their efficacy for strain quantitation and tracking.

7.1.5. COLLABORATIVE RESEARCH EXTENDING THE METHODS AND SKILLS DEVELOPED WITHIN THIS PROJECT

During this study, a feeding trial using the Cultech Lab4® probiotic supplement was ongoing in healthy human male adults. To rapidly and cost-effectively profile the bacterial diversity of faecal samples taken from this study, Ribosomal Intergenic Spacer Analysis (RISA) was trialled as a method for examining the variable intergenic spacer region of the bacterial ribosomal operon. The profiles generated were compared in a similar approach to the RAPD profile generated in Chapter 2. The exceptionally high bacterial diversity of human faeces, coupled with groups of very closely related species meant that this technique was not effective for profiling bacterial diversity of the GIT.

During the course of this PhD, an opportunity to use RISA in a much less diverse bacterial system arose. RISA was used to great effect to profile the bacterial diversity of cystic fibrosis (CF) sputum. CF sputum diversity is variable between individuals, and many of the bacteria present are undetectable by commonly used culture-based methods. Anaerobic bacteria also grow to high densities in CF sputum and these are not captured by routine aerobic culture used in standard diagnosis of CF infection. Very low diversity or monoculture sputum is associated with rapid decline in host prognosis. It is therefore vital to quickly and accurately profile the diversity of CF sputum to correctly treat the condition. The initial analysis validating the RISA could be used to qualitatively measure changes in bacteria diversity within CF sputum was published as part of:

Baxter, C. G., Rautemaa, R., Jones, A. M., Webb, A. K., Bull, M., Mahenthiralingam, E. & Denning, D. W. (2013) Intravenous antibiotics reduce the presence of *Aspergillus* in adult cystic fibrosis sputum. Thorax, 68 (7) 652-657.

A copy of this publication is included as Appendix 2 and further studies using the RISA method to examine CF infection are in preparation.

8. REFERENCES

Achtman, M. (2008) Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. *Annual Review of Microbiology,* **62** 53-70.

Ahrné, S., Nobaek, S., Jeppsson, B., Adlerberth, I., Wold, A. E. & Molin, G. (1998) The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. *Journal of Applied Microbiology,* **85** (1) 88-94.

Allen, S., Jordan, S., Storey, M., Thornton, C., Gravenor, M., Garaiova, I., Jones, R., Macfarlane, T., Seager, A., Manshian, B., Moller, M., Omakobia, M., Plummer, S., Wang, D. & Morgan, G. (2012) Probiotics and atopic eczema: a double-blind randomised controlled trial. *Archives of Disease in Childhood,* **97** (Suppl 1) A2.

Altermann, E. & Klaenhammer, T. R. (2011) Group-specific comparison of four lactobacilli isolated from human sources using differential blast analysis. *Genes & Nutrition,* **6** (3) 319-340.

Altermann, E., Russell, W. M., Azcarate-Peril, M. A., Barrangou, R., Buck, B. L., McAuliffe, O., Souther, N., Dobson, A., Duong, T., Callanan, M., Lick, S., Hamrick, A., Cano, R. & Klaenhammer, T. R. (2005) Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. *Proceedings of the National Academy of Sciences of the United States of America,* **102** (11) 3906-3912.

Angelakis, E., Million, M., Henry, M. & Raoult, D. (2011) Rapid and accurate bacterial identification in probiotics and yoghurts by MALDI-TOF mass spectrometry. *Journal of Food Science,* **76** (8) M568-572.

Angiuoli, S. V. & Salzberg, S. L. (2011) Mugsy: fast multiple alignment of closely related whole genomes. *Bioinformatics,* **27** (3) 334-342.

Anukam, K. C. & Reid, G. (eds.) 2007. *Probiotics: 100 years (1907-2007) after Elie Metchnikoff's observations,* Spain: Formatex.org.

Archibald, F. S. & Fridovich, I. (1981) Manganese, superoxide dismutase, and oxygen tolerance in some lactic acid bacteria. *Journal of Bacteriology,* **146** (3) 928-936.

Arnold, R. J., Karty, J. A., Ellington, A. D. & Reilly, J. P. (1999) Monitoring the growth of a bacteria culture by MALDI-MS of whole cells. *Analytical Chemistry,* **71** (10) 1990-1996.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., Fernandes, G. R., Tap, J., Bruls, T., Batto, J.-M., Bertalan, M., Borruel, N., Casellas, F., Fernandez, L., Gautier, L., Hansen, T., Hattori, M., Hayashi, T., Kleerebezem, M., Kurokawa, K., Leclerc, M., Levenez, F., Manichanh, C., Nielsen, H. B., Nielsen, T., Pons, N., Poulain, J., Qin, J., Sicheritz-Ponten, T., Tims, S., Torrents, D., Ugarte, E., Zoetendal, E. G., Wang, J., Guarner, F., Pedersen, O., de Vos, W. M., Brunak, S., Dore, J., Meta, H. I. T. C., Weissenbach, J., Erlich, S. D. & Bork, P. (2011) Enterotypes of the human gut microbiome. *Nature,* **473** (7346) 174-180.

Asakuma, S., Urashima, T., Akahori, M., Obayashi, H., Nakamura, T., Kimura, K., Watanabe, Y., Arai, I. & Sanai, Y. (2008) Variation of major neutral oligosaccharides levels in human colostrum. *European Journal of Clinical Nutrition,* **62** (4) 488-494.

Azcarate-Peril, M. A., Altermann, E., Hoover-Fitzula, R. L., Cano, R. J. & Klaenhammer, T. R. (2004) Identification and inactivation of genetic loci involved with *Lactobacillus acidophilus* acid tolerance. *Applied and Environmental Microbiology,* **70** (9) 5315-5322.

Azcarate-Peril, M. A., McAuliffe, O., Altermann, E., Lick, S., Russell, W. M. & Klaenhammer, T. R. (2005) Microarray analysis of a two-component regulatory system involved in acid resistance and proteolytic activity in *Lactobacillus acidophilus*. *Applied and Environmental Microbiology,* **71** (10) 5794-5804.

Azcarate-Peril, M. A., Tallon, R. & Klaenhammer, T. R. (2009) Temporal gene expression and probiotic attributes of *Lactobacillus acidophilus* during growth in milk. *Journal of Dairy Science,* **92** (3) 870-886.

Aznar, R. & Chenoll, E. (2006) Intraspecific diversity of *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Lactobacillus sakei*, and *Leuconostoc mesenteroides* associated with vacuum-packed meat product spoilage analyzed by randomly amplified polymorphic DNA PCR. *Journal of Food Protection,* **69** (10) 2403-2410.

Bahaka, D., Neut, C., Khattabi, A., Monget, D. & Gavini, F. (1993) Phenotypic and genomic analyses of human strains belonging or related to *Bifidobacterium longum*, *Bifidobacterium infantis*, and *Bifidobacterium breve*. *International Journal of Systematic Bacteriology,* **43** (3) 565-573.

Baldwin, A., Mahenthiralingam, E., Thickett, K. M., Honeybourne, D., Maiden, M. C. J., Govan, J. R., Speert, D. P., LiPuma, J. J., Vandamme, P. & Dowson, C. G. (2005) Multilocus sequence typing scheme that provides both species and strain differentiation for the *Burkholderia cepacia* complex. *Journal of Clinical Microbiology,* **43** (9) 4665-4673.

Barrangou, R., Azcarate-Peril, M. A., Duong, T., Conners, S. B., Kelly, R. M. & Klaenhammer, T. R. (2006) Global analysis of carbohydrate utilization by *Lactobacillus acidophilus* using cDNA microarrays. *Proceedings of the National Academy of Sciences of the United States of America,* **103** (10) 3816-3821.

Barrangou, R., Briczinski, E. P., Traeger, L. L., Loquasto, J. R., Richards, M., Horvath, P., Coûté-Monvoisin, A.-C., Leyer, G., Rendulic, S., Steele, J. L., Broadbent, J. R., Oberg, T., Dudley, E. G., Schuster, S., Romero, D. A. & Roberts, R. F. (2009) Comparison of the complete genome sequences of *Bifidobacterium animalis* subsp. *lactis* DSM 10140 and Bl-04. *Journal of Bacteriology,* **191** (13) 4144-4151.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D. A. & Horvath, P. (2007) CRISPR provides acquired resistance against viruses in prokaryotes. *Science,* **315** (5819) 1709-1712.

Bartosch, S., Woodmansey, E. J., Paterson, J. C. M., McMurdo, M. E. T. & Macfarlane, G. T. (2005) Microbiological effects of consuming a synbiotic containing *Bifidobacterium bifidum*, *Bifidobacterium lactis*, and oligofructose in elderly persons, determined by real-time polymerase chain reaction and counting of viable bacteria. *Clinical Infectious Diseases,* **40** (1) 28-37.

Bennett, J. S., Jolley, K. A., Earle, S. G., Corton, C., Bentley, S. D., Parkhill, J. & Maiden, M. C. (2012) A genomic approach to bacterial taxonomy: an examination and proposed reclassification of species within the genus *Neisseria*. *Microbiology,* **158** (Part 6) 1570-1580.

Berger, B., Pridmore, R. D., Barretto, C., Delmas-Julien, F., Schreiber, K., Arigoni, F. & Brussow, H. (2007) Similarity and differences in the *Lactobacillus acidophilus* group identified by polyphasic analysis and comparative genomics. *Journal of Bacteriology,* **189** (4) 1311-1321.

Bernardeau, M., Guguen, M. & Vernoux, J. P. (2006) Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. *FEMS Microbiology Reviews,* **30** (4) 487-513.

Bested, A., Logan, A. & Selhub, E. (2013) Intestinal microbiota, probiotics and mental health: from Metchnikoff to modern advances: Part I - autointoxication revisited. *Gut Pathogens,* **5** (1) 5.

Blaiotta, G., Fusco, V., Ercolini, D., Aponte, M., Pepe, O. & Villani, F. (2008) *Lactobacillus* strain diversity based on partial *hsp60* gene sequences and design of PCR-restriction fragment length polymorphism assays for species identification and differentiation. *Applied and Environmental Microbiology,* **74** (1) 208-215.

Blaut, M., Collins, M. D., Welling, G. W., Doré, J., van Loo, J. & de Vos, W. (2002) Molecular biological methods for studying the gut microbiota: the EU human gut flora project. *British Journal of Nutrition,* **87** (Supplement 2) 203-211.

Bolotin, A., Quinquis, B., Renault, P., Sorokin, A., Ehrlich, S. D., Kulakauskas, S., Lapidus, A., Goltsman, E., Mazur, M., Pusch, G. D., Fonstein, M., Overbeek, R., Kyprides, N., Purnelle, B., Prozzi, D., Ngui, K., Masuy, D., Hancy, F., Burteau, S., Boutry, M., Delcour, J., Goffeau, A. & Hols, P. (2004) Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. *Nature Biotechnology,* **22** (12) 1554-1558.

Bottacini, F., Dal Bello, F., Turroni, F., Milani, C., Duranti, S., Foroni, E., Viappiani, A., Strati, F., Mora, D., van Sinderen, D. & Ventura, M. (2011) Complete genome sequence of *Bifidobacterium animalis* subsp. *lactis* BLC1. *Journal of Bacteriology,* **193** (22) 6387-6388.

Bottacini, F., Medini, D., Pavesi, A., Turroni, F., Foroni, E., Riley, D., Giubellini, V., Tettelin, H., van Sinderen, D. & Ventura, M. (2010) Comparative genomics of the genus *Bifidobacterium*. *Microbiology,* **156** (11) 3243-3254.

Bottacini, F., Milani, C., Turroni, F., Sánchez, B., Foroni, E., Duranti, S., Serafini, F., Viappiani, A., Strati, F., Ferrarini, A., Delledonne, M., Henrissat, B., Coutinho, P., Fitzgerald, G. F., Margolles, A., van Sinderen, D. & Ventura, M. (2012) *Bifidobacterium asteroides* PRL2011 genome analysis reveals clues for colonization of the insect gut. *PLoS ONE,* **7** (9) e44229.

Boyd, M. A., Antonio, M. A. & Hillier, S. L. (2005) Comparison of API 50 CH strips to whole-chromosomal DNA probes for identification of *Lactobacillus* species. *Journal of Clinical Microbiology,* **43** (10) 5309-5311.

Brenner, S. (2010) Sequences and consequences. *Philosophical Transactions of the Royal Society B: Biological Sciences,* **365** (1537) 207-212.

Briczinski, E. P., Loquasto, J. R., Barrangou, R., Dudley, E. G., Roberts, A. M. & Roberts, R. F. (2009) Strain-specific genotyping of *Bifidobacterium animalis* subsp. *lactis* by using single-nucleotide polymorphisms, insertions, and deletions. *Applied and Environmental Microbiology,* **75** (23) 7501-7508.

Briczinski, E. P. & Roberts, R. F. (2006) Technical note: a rapid pulsed-field gel electrophoresis method for analysis of bifidobacteria. *Journal of Dairy Science,* **89** (7) 2424-2427.

Brigidi, P., Vitali, B., Swennen, E., Bazzocchi, G. & Matteuzzi, D. (2001) Effects of probiotic administration upon the composition and enzymatic activity of human fecal microbiota in patients with irritable bowel syndrome or functional diarrhea. *Research in Microbiology,* **152** (8) 735-741.

Bron, P. A., van Baarlen, P. & Kleerebezem, M. (2012) Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa. *Nature Reviews Microbiology,* **10** (1) 66-78.

Brooks, S. P., McAllister, M., Sandoz, M. & Kalmokoff, M. L. (2003) Culture-independent phylogenetic analysis of the faecal flora of the rat. *Canadian Journal of Microbiology,* **49** (10) 589-601.

Bruker Daltonik GmbH (2010) Application note #MT-80 microorganism identification and classification based on MALDI-TOF MS fingerprinting with MALDI biotyper.

Brüssow, H. (2001) Phages of dairy bacteria. *Annual Review of Microbiology,* **55** 283-303.

Bryant, D. & Moulton, V. (2004) Neighbor-net: an agglomerative method for the construction of phylogenetic networks. *Molecular Biology and Evolution,* **21** (2) 255-265.

Buck, B. L., Altermann, E., Svingerud, T. & Klaenhammer, T. R. (2005) Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. *Applied and Environmental Microbiology,* **71** (12) 8344-8351.

Bull, M. J., Marchesi, J. R., Vandamme, P., Plummer, S. & Mahenthiralingam, E. (2012) Minimum taxonomic criteria for bacterial genome sequence depositions and announcements. *Journal of Microbiological Methods,* **89** (1) 18-21.

Cachat, E. & Priest, F. G. (2005) *Lactobacillus suntoryeus* sp. nov., isolated from malt whisky distilleries. *International Journal of Systematic and Evolutionary Microbiology,* **55** (1) 31-34.

Cai, H., Rodriguez, B. T., Zhang, W., Broadbent, J. R. & Steele, J. L. (2007) Genotypic and phenotypic characterization of *Lactobacillus casei* strains isolated from different ecological niches suggests frequent recombination and niche specificity. *Microbiology,* **153** (8) 2655-2665.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K. & Madden, T. (2009) BLAST+: architecture and applications. *BMC Bioinformatics,* **10** (1) 421.

Campbell, M., Mahenthiralingam, E. & Speert, D. P. (2000) Evaluation of random amplified polymorphic DNA typing of *Pseudomonas aeruginosa*. *Journal of Clinical Microbiology,* **38** (12) 4614-4615.

Candela, M., Vitali, B., Matteuzzi, D. & Brigidi, P. (2004) Evaluation of the rrn operon copy number in *Bifidobacterium* using real-time PCR. *Letters in Applied Microbiology,* **38** (3) 229-232.

Carr, F. J., Chill, D. & Maida, N. (2002) The lactic acid bacteria: A literature survey. *Critical Reviews in Microbiology,* **28** 281-370.

Carver, T. J., Rutherford, K. M., Berriman, M., Rajandream, M.-A., Barrell, B. G. & Parkhill, J. (2005) ACT: the Artemis comparison tool. *Bioinformatics,* **21** (16) 3422-3423.

Cheplin, H. A. & Rettger, L. F. (1920) Studies on the transformation of the intestinal flora, with special reference to the implantation of *Bacillus acidophilus*: II. Feeding experiments on man. *Proceedings of the National Academy of Sciences of the United States of America,* **6** (12) 704-705.

Chervaux, C., Grimaldi, C., Bolotin, A., Quinquis, B., Legrain-Raspaud, S., van Hylckama Vlieg, J. E. T., Denariaz, G. & Smokvina, T. (2011) Genome sequence of the probiotic strain *Bifidobacterium animalis* subsp. *lactis* CNCM I-2494. *Journal of Bacteriology,* **193** (19) 5560-5561.

Chung, Y.-J., Kim, S.-K. & Choi, E.-C. (1997) The effects of *Bifidobacterium bifidum* OFR9, a strain resistant to antituberculosis and antileprosy agents, on fecal flora in mice. *The Journal of General and Applied Microbiology,* **43** (1) 61-66.

Claesson, M. J., van Sinderen, D. & O'Toole, P. W. (2007) The genus *Lactobacillus* - a genomic basis for understanding its diversity. *FEMS Microbiology Letters,* **269** (3) 22-28.

Claesson, M. J., van Sinderen, D. & O'Toole, P. W. (2008) *Lactobacillus* phylogenomics - towards a reclassification of the genus. *International Journal of Systematic and Evolutionary Microbiology,* **58** (12) 2945-2954.

Clark, C. G., Grant, C. C., Pollari, F., Marshall, B., Moses, J., Tracz, D. M. & Gilmour, M. W. (2012) Effects of the *Campylobacter jejuni* CJIE1 prophage homologs on adherence and invasion in culture, patient symptoms, and source of infection. *BMC Microbiology,* **12** 269.

Cody, A. J., McCarthy, N. D., Jansen van Rensburg, M., Isinkaye, T., Bentley, S. D., Parkhill, J., Dingle, K. E., Bowler, I. C., Jolley, K. A. & Maiden, M. C. (2013) Real-time genomic epidemiological evaluation of human *Campylobacter* isolates by use of whole-genome multilocus sequence typing. *Journal of Clinical Microbiology,* **51** (8) 2526-2534.

Coenye, T., Gevers, D., Van de Peer, Y., Vandamme, P. & Swings, J. (2005) Towards a prokaryotic genomic taxonomy. *FEMS microbiology Reviews,* **29** (2) 147-167.

Coenye, T. & Vandamme, P. (2003) Extracting phylogenetic information from whole-genome sequencing projects: the lactic acid bacteria as a test case. *Microbiology,* **149** (Pt 12) 3507-3517.

Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., Kulam-Syed-Mohideen, A. S., McGarrell, D. M., Marsh, T., Garrity, G. M. & Tiedje, J. M. (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Research,* **37** (Database issue) D141-145.

Collins, M. D., Rodrigues, U., Ash, C., Aguirre, M., Farrow, J. A. E., Martinez-Murcia, A., Phillips, B. A., Williams, A. M. & Wallbanks, S. (1991) Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. *FEMS Microbiology Letters,* **77** (1) 5-12.

Cooper, I. R., White, J., Mahenthiralingam, E. & Hanlon, G. W. (2008) Long-term persistence of a single *Legionella pneumophila* strain possessing the mip gene in a municipal shower despite repeated cycles of chlorination. *The Journal of hospital infection,* **70** (2) 154-159.

Dawyndt, P., Vancanneyt, M., de Meyer, H. & Swings, J. (2005) Knowledge accumulation and resolution of data inconsistencies during the integration of microbial information sources. *IEEE Transactions on Knowledge and Data Engineering,* **17** (8) 1111-1126.

de Bruijn, F. J. (1992) Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Applied and Environmental Microbiology,* **58** (7) 2180-2187.

De Bruyne, K., Slabbinck, B., Waegeman, W., Vauterin, P., De Baets, B. & Vandamme, P. (2011) Bacterial species identification from MALDI-TOF mass spectra through data analysis and machine learning. *Systematic and Applied Microbiology,* **34** (1) 20-29.

De Dea Lindner, J., Canchaya, C., Zhang, Z., Neviani, E., Fitzgerald, G. F., van Sinderen, D. & Ventura, M. (2007) Exploiting *Bifidobacterium* genomes: The molecular basis of stress response. *International Journal of Food Microbiology,* **120** (1–2) 13-24.

de las Rivas, B., Marcobal, A. & Munoz, R. (2006) Development of a multilocus sequence typing method for analysis of *Lactobacillus plantarum* strains. *Microbiology,* **152** (1) 85-93.

de Man, J. C., Rogosa, M. & Sharpe, M. E. (1960) A medium for the cultivation of lactobacilli. *Journal of Applied Microbiology,* **23** 130-135.

de Vries, W. & Stouthamer, A. H. (1967) Pathway of glucose fermentation in relation to the taxonomy of bifidobacteria. *Journal of Bacteriology,* **93** (2) 574-576.

de Vries, W. & Stouthamer, A. H. (1968) Fermentation of glucose, lactose, galactose, mannitol, and xylose by bifidobacteria. *Journal of Bacteriology,* **96** (2) 472-478.

Demirci, A. & Pometto, A., III (1992) Enhanced production of d(−)-lactic acid by mutants of *Lactobacillus delbrueckii AT*CC 9649. *Journal of Industrial Microbiology,* **11** (1) 23-28.

Desvaux, M., Hebraud, M., Talon, R. & Henderson, I. R. (2009) Secretion and subcellular localizations of bacterial proteins: a semantic awareness issue. *Trends in Microbiology,* **17** (4) 139-145.

Díez-Villaseñor, C., Almendros, C., García-Martínez, J. & Mojica, F. J. M. (2010) Diversity of CRISPR loci in *Escherichia coli*. *Microbiology,* **156** (5) 1351-1361.

Douillard, F. P., Kant, R., Ritari, J., Paulin, L., Palva, A. & de Vos, W. M. (2013) Comparative genome analysis of *Lactobacillus casei* strains isolated from Actimel and Yakult products reveals marked similarities and points to a common origin. *Microbial Biotechnology,* **6** (5) 576-587.

Du Plessis, E. M. & Dicks, L. M. T. (1995) Evaluation of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus acidophilus*, *Lactobacillus crispatus*, *Lactobacillus amylovorus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, and *Lactobacillus johnsonii*. *Current Microbiology,* **31** (2) 114-118.

Dušková, M., Šedo, O., Kšicová, K., Zdráhal, Z. & Karpíšková, R. (2012) Identification of lactobacilli isolated from food by genotypic methods and MALDI-TOF MS. *International Journal of Food Microbiology,* **159** (2) 107-114.

Dykes, G. A. & von Holy, A. (1994) Strain typing in the genus *Lactobacillus*. *Letters in Applied Microbiology,* **19** (2) 63-66.

Eisen, J. A. (1995) The RecA protein as a model molecule for molecular systematic studies of bacteria: comparison of trees of RecAs and 16S rRNAs from the same species. *Journal of Molecular Evolution,* **41** (6) 1105-1123.

Euzéby, J. P. (1997) List of bacterial names with standing in nomenclature: a folder available on the internet. *International Journal of Systematic Bacteriology,* **47** (2) 590-592.

FAO/WHO. Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria. Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report, 2001 Cordoba, Argentina. 1-34.

FAO/WHO (2002) Guidelines for the evaluation of probiotics in food. *Food and Agriculture Organization of the United Nations and World Health Organization Working Group Report.* London, Ontario.

Farnworth, E. R. (2008) The evidence to support health claims for probiotics. *Journal of Nutrition,* **138** (6) 1250-1254.

Felis, G. E. & Dellaglio, F. (2007) Taxonomy of lactobacilli and bifidobacteria. *Current Issues in Intestinal Microbiology,* (8) 44-61.

Field, D., Garrity, G., Gray, T., Morrison, N., Selengut, J., Sterk, P., Tatusova, T., Thomson, N., Allen, M. J., Angiuoli, S. V., Ashburner, M., Axelrod, N., Baldauf, S., Ballard, S., Boore, J., Cochrane, G., Cole, J., Dawyndt, P., De Vos, P., DePamphilis, C., Edwards, R., Faruque, N., Feldman, R., Gilbert, J., Gilna, P., Glockner, F. O., Goldstein, P., Guralnick, R., Haft, D., Hancock, D., Hermjakob, H., Hertz-Fowler, C., Hugenholtz, P., Joint, I., Kagan, L., Kane, M., Kennedy, J., Kowalchuk, G., Kottmann, R., Kolker, E., Kravitz, S., Kyrpides, N., Leebens-Mack, J., Lewis, S. E., Li, K., Lister, A. L., Lord, P., Maltsev, N., Markowitz, V., Martiny, J., Methe, B., Mizrachi, I., Moxon, R., Nelson, K., Parkhill, J., Proctor, L., White, O., Sansone, S. A., Spiers, A., Stevens, R., Swift, P., Taylor, C., Tateno, Y., Tett, A., Turner, S., Ussery, D., Vaughan, B., Ward, N., Whetzel, T., San Gil, I., Wilson, G. & Wipat, A. (2008) The minimum information about a genome sequence (MIGS) specification. *Nature Biotechnology,* **26** (5) 541-547.

Foschino, R., Picozzi, C. & Galli, A. (2001) Comparative study of nine *Lactobacillus fermentum* bacteriophages. *Journal of Applied Microbiology,* **91** (3) 394-403.

Fraga, M., Scavone, P. & Zunino, P. (2005) Preventive and therapeutic administration of an indigenous *Lactobacillus* sp. strain against *Proteus mirabilis* ascending urinary tract infection in a mouse model. *Antonie Van Leeuwenhoek,* **88** (1) 25-34.

Fraser, C., Alm, E. J., Polz, M. F., Spratt, B. G. & Hanage, W. P. (2009) The bacterial species challenge: Making sense of genetic and ecological diversity. *Science,* **323** (5915) 741-746.

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., Tobe, T., Clarke, J. M., Topping, D. L., Suzuki, T., Taylor, T. D., Itoh, K., Kikuchi, J., Morita, H., Hattori, M. & Ohno, H. (2011) *Bifidobacteria* can protect from enteropathogenic infection through production of acetate. *Nature,* **469** (7331) 543-547.

Fuller, R. (1989) Probiotics in man and animals. *Journal of Applied Bacteriology,* **66** (5) 365-378.

Garrigues, C., Johansen, E. & Pedersen, M. B. (2010) Complete genome sequence of *Bifidobacterium animalis* subsp. *lactis* BB-12, a widely consumed probiotic strain. *Journal of Bacteriology,* **192** (9) 2467-2468.

Gevers, D., Cohan, F. M., Lawrence, J. G., Spratt, B. G., Coenye, T., Feil, E. J., Stackebrandt, E., Van de Peer, Y., Vandamme, P., Thompson, F. L. & Swings, J. (2005) Opinion: Re-evaluating prokaryotic species. *Nature Reviews Microbiology,* **3** (9) 733-739.

Gevers, D., Huys, G. & Swings, J. (2001) Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiology Letters,* **205** 31-36.

Gill, S. R., Pop, M., DeBoy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., Gordon, J. I., Relman, D. A., Fraser-Liggett, C. M. & Nelson, K. E. (2006) Metagenomic analysis of the human distal gut microbiome. *Science,* **312** (5778) 1355-1359.

Gilliland, S. E. (1989) Acidophilus milk products: a review of potential benefits to consumers. *Journal of Dairy Science,* **72** (10) 2483-2494.

Gilliland, S. E. & Speck, M. L. (1977) Antagonistic action of *Lactobacillus acidophilus* toward intestinal and foodborne pathogens in associative cultures. *Journal of Food Protection,* **40** 820-823.

Gopal, P. K., Prasad, J., Smart, J. & Gill, H. S. (2001) In vitro adherence properties of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*. *International Journal of Food Microbiology,* **67** (3) 207-216.

Greene, J. D. & Klaenhammer, T. R. (1994) Factors involved in adherence of lactobacilli to human Caco-2 cells. *Applied and Environmental Microbiology,* **60** (12) 4487-4494.

Gregory R. Warnes, Ben Bolker, Lodewijk Bonebakker, Robert Gentleman, Wolfgang Huber, Andy Liaw, Thomas Lumley, Martin Maechler, Arni Magnusson, Steffen Moeller, Marc Schwartz & Bill Venables (2013) gplots: Various R programming tools for plotting data.

Grigoroff, S. (1905) "Etude sur le lait fermenté comestible : le 'Kissélo-mléko' de Bulgarie". *Revue Médicale de la Suisse Romande (in French).* Geneva: Librairie de L'Université.

Grissa, I., Vergnaud, G. & Pourcel, C. (2007) The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. *BMC Bioinformatics,* **8** (1) 172.

Guarner, F. & Malagelada, J.-R. (2003) Gut flora in health and disease. *Lancet,* **361** (9356) 512-519.

Gueimonde, M., Delgado, S., Mayo, B., Ruas-Madiedo, P., Margolles, A. & de los Reyes-Gavilán, C. G. (2004) Viability and diversity of probiotic *Lactobacillus* and *Bifidobacterium* populations included in commercial fermented milks. *Food Research International,* **37** (9) 839-850.

Gueimonde, M., Ventura, M., Margolles, A. & Sánchez, B. (2012) Genome sequence of the immunomodulatory strain *Bifidobacterium bifidum* LMG 13195. *Journal of Bacteriology,* **194** (24) 6997.

Guillier, L., Pardon, P. & Augustin, J.-C. (2005) Influence of stress on individual lag time distributions of *Listeria monocytogenes*. *Applied and Environmental Microbiology,* **71** (6) 2940-2948.

Guslandi, M., Mezzi, G., Sorghi, M. & Testoni, P. A. (2000) *Saccharomyces boulardii* in maintenance treatment of Crohn's disease. *Digestive Diseases and Sciences,* **45** (7) 1462-1464.

Ham, J.-S., Lee, T., Byun, M.-J., Lee, K.-T., Kim, M.-K., Han, G.-S., Jeong, S.-G., Oh, M.-H., Kim, D.-H. & Kim, H. (2011) Complete genome sequence of *Bifidobacterium longum* subsp. *longum* KACC 91563. *Journal of Bacteriology,* **193** (18) 5044.

Hammes, W. P. & Vogel, R. F. (1995) The genus *Lactobacillus*. *In:* Wood, B. J. B. & Holzapfel, W. H. (eds.) *The Genera of Lactic Acid Bacteria.* Glasgow: Blackie Academic & Professional.

Hao, P., Zheng, H., Yu, Y., Ding, G., Gu, W., Chen, S., Yu, Z., Ren, S., Oda, M., Konno, T., Wang, S., Li, X., Ji, Z.-S. & Zhao, G. (2011a) Complete sequencing and pan-genomic analysis of *Lactobacillus delbrueckii* subsp. *bulgaricus* reveal its genetic basis for industrial yogurt production. *PLoS ONE,* **6** (1) e15964.

Hao, Y., Huang, D., Guo, H., Xiao, M., An, H., Zhao, L., Zuo, F., Zhang, B., Hu, S., Song, S., Chen, S. & Ren, F. (2011b) Complete genome sequence of *Bifidobacterium longum* subsp. *longum* BBMN68, a new strain from a healthy Chinese centenarian. *Journal of Bacteriology,* **193** (3) 787-788.

Harris, S. R., Feil, E. J., Holden, M. T., Quail, M. A., Nickerson, E. K., Chantratita, N., Gardete, S., Tavares, A., Day, N., Lindsay, J. A., Edgeworth, J. D., de Lencastre, H., Parkhill, J., Peacock, S. J. & Bentley, S. D. (2010) Evolution of MRSA during hospital transmission and intercontinental spread. *Science,* **327** (5964) 469-474.

Havenaar, R. & Huis in't Veld, J. H. J. (1992) Probiotics: A general view. *In:* Wood, B. J. B. (ed.) *The Lactic Acid Bacteria Volume 1.* Springer US.

He, F., Ouwehand, A. C., Isolauri, E., Hashimoto, H., Benno, Y. & Salminen, S. (2001) Comparison of mucosal adhesion and species identification of bifidobacteria isolated from healthy and allergic infants. *FEMS Immunology & Medical Microbiology,* **30** (1) 43-47.

Hill, J. E., Fernando, U. W. M., Zello, G. A., Tyler, R. T., Dahl, W. J. & Kessel, A. G. V. (2010a) Improvement of the representation of bifidobacteria in fecal microbiota metagenomic libraries by application of the *cpn60* universal primer cocktail. *Applied and Environmental Microbiology,* **76** (13) 4550-4552.

Hill, J. E., Fernando, W. M. U., Zello, G. A., Tyler, R. T., Dahl, W. J. & Kessel, A. G. V. (2010b) Improvement of the representation of bifidobacteria in fecal microbiota metagenomic libraries by application of the *cpn60* universal primer cocktail. *Applied and Environmental Microbiology,* **76** (13) 4550-4552.

Hirayama, K., Baranczewski, P., Akerlund, J. E., Midtvedt, T., Moller, L. & Rafter, J. (2000) Effects of human intestinal flora on mutagenicity of and DNA adduct formation from food and environmental mutagens. *Carcinogenesis,* **21** (11) 2105-2111.

Holland, D. (1920) Generic index of the commoner forms of bacteria. *Journal of Bacteriology,* **5** 215-229.

Holzapfel, W. H., Haberer, P., Geisen, R., Bjorkroth, J. & Schillinger, U. (2001) Taxonomy and important features of probiotic microorganisms in food and nutrition. *The American Journal of Clinical Nutrition,* **73** (2) 365S-373S.

Horvath, P., Romero, D. A., Coûté-Monvoisin, A.-C., Richards, M., Deveau, H., Moineau, S., Boyaval, P., Fremaux, C. & Barrangou, R. (2008) Diversity, activity, and evolution of CRISPR loci in *Streptococcus thermophilus*. *Journal of Bacteriology,* **190** (4) 1401-1412.

Huson, D. H. & Bryant, D. (2006) Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution,* **23** (2) 254-267.

Ibnou-Zekri, N., Blum, S., Schiffrin, E. J. & Weid, T. v. d. (2003) Divergent patterns of colonization and immune response elicited from two intestinal *Lactobacillus* strains that display similar properties *in vitro*. *Infection and Immunity,* **71** (1) 428-436.

Johnson, J. L., Phelps, C. F., Cummins, C. S., London, J. & Gasser, F. (1980) Taxonomy of the *Lactobacillus acidophilus* group. *International Journal of Systematic Bacteriology,* **30** (1) 53-68.

Jolley, K. A., Bliss, C. M., Bennett, J. S., Bratcher, H. B., Brehony, C., Colles, F. M., Wimalarathna, H., Harrison, O. B., Sheppard, S. K., Cody, A. J. & Maiden, M. C. (2012) Ribosomal multilocus

sequence typing: universal characterization of bacteria from domain to strain. *Microbiology,* **158** (Pt 4) 1005-1015.

Jolley, K. A. & Maiden, M. C. (2010) BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics,* **11** 595.

Jost, T., Lacroix, C., Braegger, C. P. & Chassard, C. (2012) New insights in gut microbiota establishment in healthy breast fed neonates. *PLoS ONE,* **7** (8) e44595.

Kahm, M., Hasenbrink, G., Lichtenberg-Frate, H., Ludwig, J. & Kschischo, M. (2010) {grofit}: Fitting biological growth curves with {R}. *Journal of Statistical Software,* **33** (7) 1-21.

Kankainen, M., Paulin, L., Tynkkynen, S., von Ossowski, I., Reunanen, J., Partanen, P., Satokari, R., Vesterlund, S., Hendrickx, A. P. A., Lebeer, S., De Keersmaecker, S. C. J., Vanderleyden, J., Hämäläinen, T., Laukkanen, S., Salovuori, N., Ritari, J., Alatalo, E., Korpela, R., Mattila-Sandholm, T., Lassig, A., Hatakka, K., Kinnunen, K. T., Karjalainen, H., Saxelin, M., Laakso, K., Surakka, A., Palva, A., Salusjärvi, T., Auvinen, P. & de Vos, W. M. (2009) Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human- mucus binding protein. *Proceedings of the National Academy of Sciences of the United States of America,* **106** (40) 17193-17198.

Kern, C. C., Usbeck, J. C., Vogel, R. F. & Behr, J. (2013) Optimization of matrix-assisted-laser-desorption–ionization-time-of-flight mass spectrometry for the identification of bacterial contaminants in beverages. *Journal of Microbiological Methods,* **93** (3) 185-191.

Khaleghi, M., Kasra Kermanshahi, R. & Zarkesh-Esfahani, S. H. (2011) Effects of penicillin G on morphology and certain physiological parameters of *Lactobacillus acidophilus* ATCC 4356. *Journal of Microbiology and Biotechnology,* **21** (8) 822-829.

Khaleghi, M., Kermanshahi, R. K., Yaghoobi, M. M., Zarkesh-Esfahani, S. H. & Baghizadeh, A. (2010) Assessment of bile salt effects on S-layer production, *slp* gene expression and some physicochemical properties of *Lactobacillus acidophilus* ATCC 4356. *Journal of Microbiology and Biotechnology,* **20** (4) 749-756.

Kim, J. F., Jeong, H., Yu, D. S., Choi, S.-H., Hur, C.-G., Park, M.-S., Yoon, S. H., Kim, D.-W., Ji, G. E., Park, H.-S. & Oh, T. K. (2009) Genome sequence of the probiotic bacterium *Bifidobacterium animalis* subsp. *lactis* AD011. *Journal of Bacteriology,* **191** (2) 678-679.

Klaenhammer, T. R., Barrangou, R., Buck, B. L., Azcarate-Peril, M. A. & Altermann, E. (2005) Genomic features of lactic acid bacteria effecting bioprocessing and health. *FEMS Microbiology Reviews,* **29** (3) 393-409.

Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O. P., Leer, R., Tarchini, R., Peters, S. A., Sandbrink, H. M., Fiers, M. W. E. J., Stiekema, W., Lankhorst, R. M. K., Bron, P. A., Hoffer, S. M., Groot, M. N. N., Kerkhoven, R., de Vries, M., Ursing, B., de Vos, W. M. & Siezen, R. J. (2003) Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proceedings of the National Academy of Sciences of the United States of America,* **100** (4) 1990-1995.

Kleerebezem, M. & Hugenholtz, J. (2003) Metabolic pathway engineering in lactic acid bacteria. *Current Opinion in Biotechnology,* **14** (2) 232-237.

Kleerebezem, M. & Vaughan, E. E. (2009) Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity. *Annual Review of Microbiology,* **63** 269-290.

Klein, G., Pack, A., Bonaparte, C. & Reuter, G. (1998) Taxonomy and physiology of probiotic lactic acid bacteria. *International Journal of Food Microbiology,* **41** (2) 103-125.

Klijn, A., Mercenier, A. & Arigoni, F. (2005) Lessons from the genomes of bifidobacteria. *FEMS Microbiology Reviews,* **29** (3) 491-509.

Ko, E. J., Goh, J. S., Lee, B. J., Choi, S. H. & Kim, P. H. (1999) *Bifidobacterium bifidum* exhibits a lipopolysaccharide-like mitogenic activity for murine B lymphocytes. *Journal of Dairy Science,* **82** (9) 1869-1876.

Konstantinidis, K. T. & Tiedje, J. M. (2005) Genomic insights that advance the species definition for prokaryotes. *Proceedings of the National Academy of Sciences of the United States of America,* **102** (7) 2567-2572.

Koonin, E. V. & Makarova, K. S. (2009) CRISPR-Cas: an adaptive immunity system in prokaryotes. *F1000 Biology Reports,* **1** (95).

Kopp-Hoolihan, L. (2001) Prophylactic and therapeutic uses of probiotics: A review. *Journal of the American Dietetic Association,* **101** (2) 229-241.

Korhonen, J. M., Sclivagnotis, Y. & Wright, A. v. (2007) Characterization of dominant cultivable lactobacilli and their antibiotic resistance profiles from faecal samples of weaning piglets. *Journal of Applied Microbiology,* **103** (6) 2496-2503.

Kullen, M. J., Sanozky-Dawes, R. B., Crowell, D. C. & Klaenhammer, T. R. (2000) Use of the DNA sequence of variable regions of the 16S rRNA gene for rapid and accurate identification of bacteria in the *Lactobacillus acidophilus* complex. *Journal of Applied Microbiology,* **89** (3) 511-516.

Kulp, W. L. & Rettger, L. F. (1924) Comparative study of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*. *Journal of Bacteriology,* **9** (4) 357-395.

Lacher, D. W., Steinsland, H., Blank, T. E., Donnenberg, M. S. & Whittam, T. S. (2007) Molecular evolution of typical enteropathogenic *Escherichia coli*: Clonal analysis by multilocus sequence typing and virulence gene allelic profiling. *Journal of Bacteriology,* **189** (2) 342-350.

Lane, D. J. (1991) 16S/23S rRNA sequencing. *In:* Stackebrandt, E. & Goodfellow, M. (eds.) *Nucleic Acid Techniques in Bacterial Systematics.* Chichester, UK: John Wiley and Sons.

Lee, J.-H. & O'Sullivan, D. J. (2010) Genomic insights into bifidobacteria. *Microbiology and Molecular Biology Reviews,* **74** (3) 378-416.

Lee, J. H., Karamychev, V. N., Kozyavkin, S. A., Mills, D., Pavlov, A. R., Pavlova, N. V., Polouchine, N. N., Richardson, P. M., Shakhova, V. V., Slesarev, A. I., Weimer, B. & O'Sullivan, D. J. (2008) Comparative genomic analysis of the gut bacterium *Bifidobacterium longum* reveals loci susceptible to deletion during pure culture growth. *BMC Genomics,* **9** 247.

Leroy, F. & De Vuyst, L. (2004) Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science & Technology,* **15** (2) 67-78.

Leser, T. D., Amenuvor, J. Z., Jensen, T. K., Lindecrona, R. H., Boye, M. & Møller, K. (2002) Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Applied and Environmental Microbiology,* **68** (2) 673-690.

Ley, R. E., Hamady, M., Lozupone, C., Turnbaugh, P. J., Ramey, R. R., Bircher, J. S., Schlegel, M. L., Tucker, T. A., Schrenzel, M. D., Knight, R. & Gordon, J. I. (2008) Evolution of mammals and their gut microbes. *Science,* **320** (5883) 1647-1651.

Leyer, G. J., Li, S., Mubasher, M. E., Reifer, C. & Ouwehand, A. C. (2009) Probiotic effects on cold and influenza-like symptom incidence and duration in children. *Pediatrics,* **124** (2) 172-179.

Li, H. & Durbin, R. (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics,* **25** (14) 1754-1760.

Li, W., Raoult, D. & Fournier, P.-E. (2009) Bacterial strain typing in the genomic era. *FEMS Microbiology Reviews,* **33** (5) 892-916.

Lilly, D. M. & Stillwell, R. H. (1965) Probiotics: Growth-promoting factors produced by microorganisms. *Science,* **147** (3659) 747-748.

Link-Amster, H., Rochat, F., Saudan, K. y., Mignot, O. & Aeschlimann, J. m. (1994) Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. *FEMS Immunology and Medical Microbiology,* **10** (1) 55-63.

Liu, H., Du, Z., Wang, J. & Yang, R. (2007) Universal sample preparation method for characterization of bacteria by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Applied and Environmental Microbiology,* **73** (6) 1899-1907.

Lobry, J. R. & Sueoka, N. (2002) Asymmetric directional mutation pressures in bacteria. *Genome Biology,* **3** (10) RESEARCH0058.

Loquasto, J. R., Barrangou, R., Dudley, E. G. & Roberts, R. F. (2011) Short communication: The complete genome sequence of *Bifidobacterium animalis* subspecies *animalis* ATCC 25527T and comparative analysis of growth in milk with *B. animalis* subspecies *lactis* DSM 10140T. *Journal of Dairy Science,* **94** (12) 5864-5870.

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J. J. & Lee, M. D. (2003) Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Applied and Environmental Microbiology,* **69** (11) 6816-6824.

Lukjancenko, O., Ussery, D. W. & Wassenaar, T. M. (2011) Comparative genomics of *Bifidobacterium*, *Lactobacillus* and related probiotic genera. *Microbial ecology,* **63** (3) 651-673.

Luyer, M. D., Buurman, W. A., Hadfoune, M., Speelmans, G., Knol, J., Jacobs, J. A., Dejong, C. H., Vriesema, A. J. & Greve, J. W. (2005) Strain-specific effects of probiotics on gut barrier integrity following hemorrhagic shock. *Infection and Immunity,* **73** (6) 3686-3692.

Mahenthiralingam, E., Campbell, M., Foster, J., Lam, J. & Speert, D. (1996a) Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. *Journal of Clinical Microbiology,* **34** (5) 1129-1135.

Mahenthiralingam, E., Campbell, M. E., Foster, J., Lam, J. S. & Speert, D. P. (1996b) Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. *Journal of Clinical Microbiology,* **34** (5) 1129-1135.

Mahenthiralingam, E., Marchbank, A., Drevinek, P., Garaiova, I. & Plummer, S. (2009) Use of colony-based bacterial strain typing for tracking the fate of *Lactobacillus* strains during human consumption. *BMC Microbiology,* **9** (251) 251.

Mahony, J., Ainsworth, S., Stockdale, S. & van Sinderen, D. (2012) Phages of lactic acid bacteria: the role of genetics in understanding phage-host interactions and their co-evolutionary processes. *Virology,* **434** 143-150.

Maiden, M. C. (2006) Multilocus sequence typing of bacteria. *Annual Review of Microbiology,* **60** 561-588.

Maiden, M. C. J., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D. A., Feavers, I. M., Achtman, M. & Spratt, B. G. (1998) Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences of the United States of America,* **95** (6) 3140-3145.

Mainville, I., Robert, N., Lee, B. & Farnworth, E. R. (2006) Polyphasic characterization of the lactic acid bacteria in kefir. *Systematic and Applied Microbiology,* **29** (1) 59-68.

Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D. M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J. H., Diaz-Muniz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'Sullivan, D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B. & Mills, D. (2006) Comparative genomics of the lactic acid bacteria. *Proceedings of the National Academy of Sciences of the United States of America,* **103** 15611-15616.

Makarova, K. S., Haft, D. H., Barrangou, R., Brouns, S. J. J., Charpentier, E., Horvath, P., Moineau, S., Mojica, F. J. M., Wolf, Y. I., Yakunin, A. F., van der Oost, J. & Koonin, E. V. (2011) Evolution and classification of the CRISPR–Cas systems. *Nature Reviews Microbiology,* **9** (6) 467-477.

Markowitz, V. M., Chen, I. M., Palaniappan, K., Chu, K., Szeto, E., Grechkin, Y., Ratner, A., Anderson, I., Lykidis, A., Mavromatis, K., Ivanova, N. N. & Kyrpides, N. C. (2010) The integrated microbial genomes system: an expanding comparative analysis resource. *Nucleic Acids Research,* **38** (Database issue) D382-390.

Masco, L., Huys, G., De Brandt, E., Temmerman, R. & Swings, J. (2005) Culture-dependent and culture-independent qualitative analysis of probiotic products claimed to contain bifidobacteria. *International Journal of Food Microbiology,* **102** (2) 221-230.

Matsuki, T., Watanabe, K. & Tanaka, R. (2003) Genus- and species-specific PCR primers for the detection and identification of bifidobacteria. *Current Issues in Intestinal Microbiology,* **4** (2) 61-69.

Matsuki, T., Watanabe, K., Tanaka, R. & Oyaizu, H. (1998) Rapid identification of human intestinal bifidobacteria by 16S rRNA-targeted species- and group-specific primers. *FEMS Microbiology Letters,* **167** (2) 113-121.

Maukonen, J., Matto, J., Wirtanen, G., Raaska, L., Mattila-Sandholm, T. & Saarela, M. (2003) Methodologies for the characterization of microbes in industrial environments: a review. *Journal of Industrial Microbiology and Biotechnology,* **30** (6) 327-356.

McCartney, A. L., Wenzhi, W. & Tannock, G. W. (1996) Molecular analysis of the composition of the bifidobacterial and *Lactobacillus* microflora of humans. *Applied and Environmental Microbiology,* **62** (12) 4608-4613.

Medini, D., Serruto, D., Parkhill, J., Relman, D. A., Donati, C., Moxon, R., Falkow, S. & Rappuoli, R. (2008) Microbiology in the post-genomic era. *Nature Reviews Microbiology,* **6** (6) 419-430.

Mende, D. R., Sunagawa, S., Zeller, G. & Bork, P. (2013) Accurate and universal delineation of prokaryotic species. *Nature Methods,* **10** (9) 881-884.

Metchnikoff, E. (1907) Lactic acid as inhibiting intestinal putrefaction. *In:* Mitchell, P. C. (ed.) *The prolongation of life: optimistic studies.* London: Heinemann.

Milani, C., Duranti, S., Lugli, G. A., Bottacini, F., Strati, F., Arioli, S., Foroni, E., Turroni, F., van Sinderen, D. & Ventura, M. (2013) Comparative genomics of *Bifidobacterium animalis* subsp. *lactis* reveals a strict monophyletic bifidobacterial taxon. *Applied and Environmental Microbiology,* **79** (14) 4304-4315.

Miyake, T., Watanabe, K., Watanabe, T. & Oyaizu, H. (1998) Phylogenetic analysis of the genus *Bifidobacterium* and related genera based on 16S rDNA sequences. *Microbiology and Immunology,* **42** (10) 661-667.

Molenaar, D., Bringel, F., Schuren, F. H., de Vos, W. M., Siezen, R. J. & Kleerebezem, M. (2005) Exploring *Lactobacillus plantarum* genome diversity by using microarrays. *Journal of Bacteriology,* **187** (17) 6119-6127.

Moore, E. R., Mihaylova, S. A., Vandamme, P., Krichevsky, M. I. & Dijkshoorn, L. (2010) Microbial systematics and taxonomy: relevance for a microbial commons. *Research in Microbiology,* **161** (6) 430-438.

Morishita, T., Deguchi, Y., Yajima, M., Sakurai, T. & Yura, T. (1981) Multiple nutritional requirements of lactobacilli: genetic lesions affecting amino acid biosynthetic pathways. *Journal of Bacteriology,* **148** (1) 64-71.

Moro, E. (1900) Ueber die nach Gram farbbaren bacillen des säuglingsstuhles. *Wiener klinische Wochenschrift,* **13** 114-115.

Murphy, E. F., Cotter, P. D., Healy, S., Marques, T. M., O'Sullivan, O., Fouhy, F., Clarke, S. F., O'Toole, P. W., Quigley, E. M., Stanton, C., Ross, P. R., O'Doherty, R. M. & Shanahan, F. (2010) Composition and energy harvesting capacity of the gut microbiota: relationship to diet, obesity and time in mouse models. *Gut,* **59** (12) 1635-1642.

Naser, S. M., Dawyndt, P., Hoste, B., Gevers, D., Vandemeulebroecke, K., Cleenwerck, I., Vancanneyt, M. & Swings, J. (2007) Identification of lactobacilli by *pheS* and *rpoA* gene sequence analyses. *International Journal of Systematic and Evolutionary Microbiology,* **57** 2777-2789.

Naser, S. M., Hagen, K. E., Vancanneyt, M., Cleenwerck, I., Swings, J. & Tompkins, T. A. (2006) *Lactobacillus suntoryeus* Cachat and Priest 2005 is a later synonym of *Lactobacillus helveticus* (Orla-Jensen 1919) Bergey et al. 1925 (Approved Lists 1980). *International Journal of Systematic and Evolutionary Microbiology,* **56** (2) 355-360.

Neuwirth, E. (2011) RColorBrewer: ColorBrewer palettes.

Nicolas, P., Bessières, P., Ehrlich, S. D., Maguin, E. & van de Guchte, M. (2007) Extensive horizontal transfer of core genome genes between two *Lactobacillus* species found in the gastrointestinal tract. *BMC Evolutionary Biology,* **7** (1).

O'Connell Motherway, M., Zomer, A., Leahy, S. C., Reunanen, J., Bottacini, F., Claesson, M. J., O'Brien, F., Flynn, K., Casey, P. G., Munoz, J. A., Kearney, B., Houston, A. M., O'Mahony, C., Higgins, D. G., Shanahan, F., Palva, A., de Vos, W. M., Fitzgerald, G. F., Ventura, M., O'Toole, P. W. & van Sinderen, D. (2011) Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. *Proceedings of the National Academy of Sciences of the United States of America,* **108** (27) 11217-11222.

O'Sullivan, O., O'Callaghan, J., Sangrador-Vegas, A., McAuliffe, O., Slattery, L., Kaleta, P., Callanan, M., Fitzgerald, G. F., Ross, R. P. & Beresford, T. (2009) Comparative genomics of lactic acid bacteria reveals a niche-specific gene set. *BMC Microbiology,* **9** 50.

Oh, S., Roh, H., Ko, H. J., Kim, S., Kim, K. H., Lee, S. E., Chang, I. S., Kim, S. & Choi, I. G. (2011) Complete genome sequencing of *Lactobacillus acidophilus* 30SC, isolated from swine intestine. *Journal of Bacteriology,* **193** (11) 2882-2883.

Okoro, C. K., Kingsley, R. A., Connor, T. R., Harris, S. R., Parry, C. M., Al-Mashhadani, M. N., Kariuki, S., Msefula, C. L., Gordon, M. A., de Pinna, E., Wain, J., Heyderman, R. S., Obaro, S., Alonso, P. L., Mandomando, I., MacLennan, C. A., Tapia, M. D., Levine, M. M., Tennant, S. M., Parkhill, J. & Dougan, G. (2012) Intracontinental spread of human invasive *Salmonella* Typhimurium pathovariants in sub-Saharan Africa. *Nature Genetics,* **44** (11) 1215-1221.

Ouwehand, A. C., Salminen, S. & Isolauri, E. (2002) Probiotics: an overview of beneficial effects. *Antonie Van Leeuwenhoek,* **82** (1-4) 279-289.

Paineau, D., Carcano, D., Leyer, G., Darquy, S., Alyanakian, M.-A., Simoneau, G., Bergmann, J.-F., Brassart, D., Bornet, F. & Ouwehand, A. C. (2008) Effects of seven potential probiotic strains on specific immune responses in healthy adults: a double-blind, randomized, controlled trial. *FEMS Immunology & Medical Microbiology,* **53** (1) 107-113.

Palmer, C., Bik, E. M., DiGiulio, D. B., Relman, D. A. & Brown, P. O. (2007) Development of the human infant intestinal microbiota. *PLoS Biology,* **5** (7) e177.

Park, J.-H., Um, J.-I., Lee, B.-J., Goh, J.-S., Park, S.-Y., Kim, W.-S. & Kim, P.-H. (2002) Encapsulated *Bifidobacterium bifidum* potentiates intestinal IgA production. *Cellular Immunology,* **219** (1) 22-27.

Parker, R. B. (1974) Probiotics, the other half of the antibiotic story. *Animal Nutritional Health,* (29) 4-8.

Passerini, D., Beltramo, C., Coddeville, M., Quentin, Y., Ritzenthaler, P., Daveran-Mingot, M.-L. & Le Bourgeois, P. (2010) Genes but not genomes reveal bacterial domestication of *Lactococcus Lactis*. *PLoS ONE,* **5** (12) e15306.

Perez, P. F., Minnaard, Y., Disalvo, E. A. & De Antoni, G. L. (1998) Surface properties of bifidobacterial strains of human origin. *Applied and Environmental Microbiololgy,* **64** (1) 21-26.

Pfeiler, E. A., Azcarate-Peril, M. A. & Klaenhammer, T. R. (2007) Characterization of a novel bile-inducible operon encoding a two-component regulatory system in *Lactobacillus acidophilus*. *Journal of Bacteriology,* **189** 4624-4634.

Pfeiler, E. A. & Klaenhammer, T. R. (2007) The genomics of lactic acid bacteria. *Trends in Microbiology,* **15** (12) 546-553.

Pfeiler, E. A. & Klaenhammer, T. R. (2009) Role of transporter proteins in bile tolerance of *Lactobacillus acidophilus*. *Applied and Environmental Microbiology,* **75** (18) 6013-6016.

Philippe, D., Favre, L., Foata, F., Adolfsson, O., Perruisseau-Carrier, G., Vidal, K., Reuteler, G., Dayer-Schneider, J., Mueller, C. & Blum, S. (2011) Bifidobacterium lactis attenuates onset of inflammation in a murine model of colitis. *World Journal of Gastroenterology,* **17** (4) 459-469.

Phillippy, A. M., Ayanbule, K., Edwards, N. J. & Salzberg, S. L. (2009) Insignia: a DNA signature search web server for diagnostic assay development. *Nucleic Acids Research,* **37** (Suppl 2) W229-W234.

Phillippy, A. M., Mason, J. A., Ayanbule, K., Sommer, D. D., Taviani, E., Huq, A., Colwell, R. R., Knight, I. T. & Salzberg, S. L. (2007) Comprehensive DNA signature discovery and validation. *PLoS Computational Biology,* **3** (5) e98.

Pimentel, L. L., Mättö, J., Malcata, F. X., Pintado, M. E. & Saarela, M. (2012) Survival of potentially probiotic enterococci in dairy matrices and in the human gastrointestinal tract. *International Dairy Journal,* **27** (1–2) 53-57.

Pineiro, M. & Stanton, C. (2007) Probiotic bacteria: Legislative framework— requirements to evidence basis. *The Journal of Nutrition,* **137** (3) 850S-853S.

Plummer, S., Weaver, M. A., Harris, J. C., Dee, P. & Hunter, J. (2004) *Clostridium difficile* pilot study: effects of probiotic supplementation on the incidence of *C. difficile* diarrhoea. *International Microbiology,* **7** (1) 59-62.

Pokusaeva, K., Fitzgerald, G. F. & van Sinderen, D. (2011) Carbohydrate metabolism in *Bifidobacteria*. *Genes & Nutrition,* **6** (3) 285-306.

Pot, B., Hertel, C., Ludwig, W., Descheemaeker, P., Kersters, K. & Schleifer, K. H. (1993) Identification and classification of *Lactobacillus acidophilus*, *L. gasseri* and *L. johnsonii* strains by SDS-PAGE and rRNA-targeted oligonucleotide probe hybridization. *Journal of General Microbiology,* **139** (3) 513-517.

Pridmore, R. D., Berger, B., Desiere, F., Vilanova, D., Barretto, C., Pittet, A.-C., Zwahlen, M.-C., Rouvet, M., Altermann, E., Barrangou, R., Mollet, B., Mercenier, A., Klaenhammer, T., Arigoni, F. & Schell, M. A. (2004) The genome sequence of the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533. *Proceedings of the National Academy of Sciences of the United States of America,* **101** (8) 2512-2517.

R Development Core Team (2012) R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Reid, G. (1999) The scientific basis for probiotic strains of *Lactobacillus*. *Applied and Environmental Microbiology,* **65** (9) 3763-3766.

Ringel-Kulka, T., Palsson, O. S., Maier, D., Carroll, I., Galanko, J. A., Leyer, G. & Ringel, Y. (2011) Probiotic bacteria *Lactobacillus acidophilus* NCFM and *Bifidobacterium lactis* Bi-07 versus placebo for the symptoms of bloating in patients with functional bowel disorders: a double-blind study. *Journal of Clinical Gastroenterology,* **45** (6) 518-525.

Roberts, E., Sethi, A., Montoya, J., Woese, C. R. & Luthey-Schulten, Z. (2008) Molecular signatures of ribosomal evolution. *Proceedings of the National Academy of Sciences of the United States of America,* **105** (37) 13953-13958.

Rogosa, M. & Sharpe, M. E. (1960) Species differentiation of human vaginal lactobacilli. *Journal of General Microbiology,* **23** (1) 197-201.

Roy, D. & Ward, P. (1990) Evaluation of rapid methods for differentiation of *Bifidobacterium* species. *Journal of Applied Microbiology,* **69** (5) 739-749.

Roy, D., Ward, P. & Champagne, G. (1996) Differentiation of bifidobacteria by use of pulsed-field gel electrophoresis and polymerase chain reaction. *International Journal of Food Microbiology,* **29** (1) 11-29.

Roy, D., Ward, P. & Vincent, D. (1999) Strain identification of probiotic *Lactobacillus casei*-related isolates with randomly amplified polymorphic DNA and pulsed-field gel electrophoresis methods. *Biotechnology Techniques,* **13** (12) 843-847.

Rozen, S. & Skaletsky, H. J. (2000) Primer3 on the www for general users and for biologist programmers. *In:* Misener, S. & Krawetz, S. A. (eds.) *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* Totowa: Humana Press Inc.

Ruiz-Moyano, S., Tao, N., Underwood, M. A. & Mills, D. A. (2012) Rapid discrimination of *Bifidobacterium animalis* subspecies by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Food Microbiology,* **30** (2) 432-437.

Saavedra, J. M., Bauman, N. A., Oung, I., Perman, J. A. & Yolken, R. H. (1994) Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhoea and shedding of rotavirus. *Lancet,* **344** (8929) 1046-1049.

Salvetti, E., Fondi, M., Fani, R., Torriani, S. & Felis, G. E. (2013) Evolution of lactic acid bacteria in the order *Lactobacillales* as depicted by analysis of glycolysis and pentose phosphate pathways. *Systematic and Applied Microbiology,* **36** (5) 291-305.

Salvetti, E., Torriani, S. & Felis, G. E. (2012) The genus *Lactobacillus*: A taxonomic update. *Probiotics and Antimicrobial Proteins,* **4** (4) 217-226.

Sanders, M. E. (1993) Summary of the conclusions from a consensus panel of experts on health attributes on lactic cultures: significance to fluid milk products containing cultures. *Journal of Dairy Science,* **76** 1819-1828.

Sanders, M. E. (2003) Probiotics: considerations for human health. *Nutrition reviews,* **61** (3) 91-99.

Sanders, M. E. (2008) Probiotics: definition, sources, selection, and uses. *Clinical Infectious Diseases,* **46** (Supplement 2) S58-S61.

Sanders, M. E. & Klaenhammer, T. R. (2001) Invited review: The scientific basis of *Lactobacillus acidophilus* NCFM functionality as a probiotic. *Journal of Dairy Science,* **84** (2) 319-331.

Sanders, M. E. & Marco, M. L. (2010) Food formats for effective delivery of probiotics. *Food Science and Technology,* **1** 65-85.

Sanders, M. E., Walker, D. C., Walker, K. M., Aoyama, K. & Klaenhammer, T. R. (1996) Performance of commercial cultures in fluid milk applications. *Journal of Dairy Science,* **79** (6) 943-955.

Sarma-Rupavtarm, R. B., Ge, Z., Schauer, D. B., Fox, J. G. & Polz, M. F. (2004) Spatial distribution and stability of the eight microbial species of the altered Schaedler flora in the mouse gastrointestinal tract. *Applied and Environmental Microbiology,* **70** (5) 2791-2800.

Sarmiento-Rubiano, L.-A., Berger, B., Moine, D., Zuniga, M., Perez-Martinez, G. & Yebra, M. (2010) Characterization of a novel *Lactobacillus* species closely related to *Lactobacillus johnsonii* using a combination of molecular and comparative genomics methods. *BMC Genomics,* **11** (1) 504.

Satokari, R. M., Vaughan, E. E., Smidt, H., Saarela, M., Mättö, J. & de Vos, W. M. (2003) Molecular approaches for the detection and identification of bifidobacteria and lactobacilli in the human gastrointestinal tract. *Systematic and Applied Microbiology,* **26** (4) 572-584.

Saxelin, M., Tynkkynen, S., Mattila-Sandholm, T. & de Vos, W. M. (2005) Probiotic and other functional microbes: from markets to mechanisms. *Current Opinion in Biotechnology,* **16** (2) 204-211.

Schaafsma, G. (1996) State of art concerning probiotic strains in milk products. *Iinternational Dairy Food Nutritional News Letters,* (5) 23-24.

Schell, M. A., Karmirantzou, M., Snel, B., Vilanova, D., Berger, B., Pessi, G., Zwahlen, M.-C., Desiere, F., Bork, P., Delley, M., Pridmore, R. D. & Arigoni, F. (2002) The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. *Proceedings of the National Academy of Sciences of the United States of America,* **99** (22) 14422-14427.

Schiffrin, E. J., Rochat, F., Link-Amster, H., Aeschlimann, J. M. & Donnet-Hughes, A. (1995) Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. *Journal of Dairy Science,* **78** (3) 491-497.

Schillinger, U., Yousif, N. M. K., Sesar, L. & Franz, C. M. A. P. (2003) Use of group-specific and RAPD-PCR analyses for rapid differentiation of *Lactobacillus* strains from probiotic yogurts. *Current Microbiology,* **47** (6) 453-456.

Schleifer, K.-H., Ehrmann, M., Beimfohr, C., Brockmann, E., Ludwig, W. & Amann, R. (1995) Application of molecular methods for the classification and identification of lactic acid bacteria. *International Dairy Journal,* **5** (8) 1081-1094.

Schleifer, K.-H. & Ludwig, W. (1995) Phylogeny of the genus *Lactobacillus* and related genera. *Systematic Applied Microbiology,* (18) 461–467.

Sela, D. A., Chapman, J., Adeuya, A., Kim, J. H., Chen, F., Whitehead, T. R., Lapidus, A., Rokhsar, D. S., Lebrilla, C. B., German, J. B., Price, N. P., Richardson, P. M. & Mills, D. A. (2008) The genome sequence of *Bifidobacterium longum* subsp. *infantis* reveals adaptations for milk utilization within the infant microbiome. *Proceedings of the National Academy of Sciences of the United States of America,* **105** (48) 18964-18969.

Sela, D. A. & Mills, D. A. (2010) Nursing our microbiota: molecular linkages between bifidobacteria and milk oligosaccharides. *Trends in Microbiology,* **18** (7) 298-307.

Shah, N. P. (2000) Probiotic bacteria: selective enumeration and survival in dairy foods. *Journal of Dairy Science,* **83** (4) 894-907.

Shah, N. P. (2007) Functional cultures and health benefits. *International Dairy Journal,* **17** (11) 1262-1277.

Shah, N. P., Lankaputhra, W. E. V., Britz, M. L. & Kyle, W. S. A. (1995) Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in commercial yoghurt during refrigerated storage. *International Dairy Journal,* **5** (5) 515-521.

Sheppard, S. K., Jolley, K. A. & Maiden, M. C. J. (2012) A gene-by-gene approach to bacterial population genomics: Whole genome MLST of *Campylobacter*. *Genes,* **3** (2) 261-277.

Simpson, P. J., Stanton, C., Fitzgerald, G. F. & Ross, R. P. (2003) Genomic diversity and relatedness of bifidobacteria isolated from a porcine cecum. *Journal of Bacteriology,* **185** (8) 2571-2581.

Sintchenko, V., Iredell, J. R. & Gilbert, G. L. (2007) Pathogen profiling for disease management and surveillance. *Nature Reviews Microbiology,* **5** (6) 464-470.

Smelt, J. P. P. M., Otten, G. D. & Bos, A. P. (2002) Modelling the effect of sublethal injury on the distribution of the lag times of individual cells of *Lactobacillus plantarum*. *International Journal of Food Microbiology,* **73** (2–3) 207-212.

Solheim, M., Aakra, A., Veb, H., Snipen, L. & Nes, I. F. (2007) Transcriptional responses of *Enterococcus faecalis* v583 to bovine bile and sodium dodecyl sulfate. *Applied and Environmental Microbiology,* **73** (18) 5767-5774.

Song, Y.-L., Kato, N., Liu, C.-X., Matsumiya, Y., Kato, H. & Watanabe, K. (2000) Rapid identification of 11 human intestinal *Lactobacillus* species by multiplex PCR assays using group- and species-specific primers derived from the 16S-23S rRNA intergenic spacer region and its flanking 23S rRNA. *FEMS Microbiology Letters,* **187** (2) 167-173.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A., Kampfer, P., Maiden, M. C., Nesme, X., Rossello-Mora, R., Swings, J., Truper, H. G., Vauterin, L., Ward, A. C. & Whitman, W. B. (2002) Report of the *ad hoc* committee for the re-evaluation of the species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology,* **52** (3) 1043-1047.

Stackebrandt, E. & Goebel, B. M. (1994) Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology,* **44** (4) 846-849.

Stahl, B. & Barrangou, R. (2012) Complete genome sequences of probiotic strains *Bifidobacterium animalis* subsp. *lactis* B420 and Bi-07. *Journal of Bacteriology,* **194** (15) 4131-4132.

Stahl, B. & Barrangou, R. (2013) Complete Genome Sequence of Probiotic Strain *Lactobacillus acidophilus* La-14. *Genome Announcements,* **1** (3).

Stanton, C., Ross, R. P., Fitzgerald, G. F. & Van Sinderen, D. (2005) Fermented functional foods based on probiotics and their biogenic metabolites. *Current Opinion in Biotechnology,* **16** (2) 198-203.

Stephenson, D. P., Moore, R. J. & Allison, G. E. (2009) Comparison and utilization of repetitive-element PCR techniques for typing *Lactobacillus* isolates from the chicken gastrointestinal tract. *Applied and Environmental Microbiology,* **75** (21) 6764-6776.

Stern, A., Keren, L., Wurtzel, O., Amitai, G. & Sorek, R. (2010) Self-targeting by CRISPR: gene regulation or autoimmunity? *Trends in Genetics,* **26** (8) 335-340.

Stiles, M. E. & Holzapfel, W. H. (1997) Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology,* **36** (1) 1-29.

Sun, Z., Chen, X., Wang, J., Gao, P., Zhou, Z., Ren, Y., Sun, T., Wang, L., Meng, H., Chen, W. & Zhang, H. (2010) Complete genome sequence of probiotic *Bifidobacterium animalis* subsp. *lactis s*train V9. *Journal of Bacteriology,* **192** (15) 4080-4081.

Tabasco, R., García-Cayuela, T., Peláez, C. & Requena, T. (2009) *Lactobacillus acidophilus* La-5 increases lactacin B production when it senses live target bacteria. *International Journal of Food Microbiology,* **132** (2-3) 109-116.

Tamime, A. Y. (2002) Fermented milks: a historical food with modern applications - a review. *European Journal of Clinical Nutrition,* **56** (Suppl 4) 2-15.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution,* **24** (8) 1596-1599.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution,* **28** (10) 2731-2739.

Taylor, G. R. & Williams, C. M. (1998) Effects of probiotics and prebiotics on blood lipids. *British Journal of Nutrition,* **80** (4) S225-230.

Tissier, H. (1900). *Recherchers sur la flora intestinale normale et pathologique du nourisson.* PhD Thesis, University of Paris.

Tomas, J., Langella, P. & Cherbuy, C. (2012) The intestinal microbiota in the rat model: major breakthroughs from new technologies. *Animal Health Research Reviews,* **13** (1) 54-63.

Tuohy, K. M., Probert, H. M., Smejkal, C. W. & Gibson, G. R. (2003) Using probiotics and prebiotics to improve gut health. *Drug Discovery Today,* **8** (15) 692-700.

Tuomola, E. M. & Salminen, S. J. (1998) Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *International Journal of Food Microbiology,* **41** (1) 45-51.

Turnbaugh, P. J., Bäckhed, F., Fulton, L. & Gordon, J. I. (2008) Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. *Cell Host & Microbe,* **3** (4) 213-223.

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R. & Gordon, J. I. (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature,* **444** (7122) 1027-1131.

Turroni, F., Bottacini, F., Foroni, E., Mulder, I., Kim, J. H., Zomer, A., Sanchez, B., Bidossi, A., Ferrarini, A., Giubellini, V., Delledonne, M., Henrissat, B., Coutinho, P., Oggioni, M., Fitzgerald, G. F., Mills, D., Margolles, A., Kelly, D., van Sinderen, D. & Ventura, M. (2010) Genome analysis of *Bifidobacterium bifidum* PRL2010 reveals metabolic pathways for host-derived glycan foraging. *Proceedings of the National Academy of Sciences of the United States of America,* **107** (45) 19514-195149.

Turroni, F., Foroni, E., Pizzetti, P., Giubellini, V., Ribbera, A., Merusi, P., Cagnasso, P., Bizzarri, B., de'Angelis, G. L., Shanahan, F., van Sinderen, D. & Ventura, M. (2009) Exploring the diversity of the bifidobacterial population in the human intestinal tract. *Applied and Environmental Microbiology,* **75** (6) 1534-1545.

Turroni, F., van Sinderen, D. & Ventura, M. (2011) Genomics and ecological overview of the genus *Bifidobacterium*. *International Journal of Food Microbiology,* **149** (1) 37-44.

Tynkkynen, S., Satokari, R., Saarela, M., Mattila-Sandholm, T. & Saxelin, M. (1999) Comparison of ribotyping, randomly amplified polymorphic DNA analysis, and pulsed-field gel electrophoresis in typing of *Lactobacillus rhamnosus* and *L. casei* strains. *Applied and Environmental Microbiology,* **65** (9) 3908-3914.

Vancanneyt, M., Huys, G., Lefebvre, K., Vankerckhoven, V., Goossens, H. & Swings, J. (2006) Intraspecific genotypic characterization of *Lactobacillus rhamnosus* strains intended for probiotic use and isolates of human origin. *Applied and Environmental Microbiology,* **72** (8) 5376-5383.

Vandamme, P., Pot, B., Gillis, M., de Vos, P., Kersters, K. & Swings, J. (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. *FEMS Microbiology Reviews,* **60** (2) 407-438.

Vankerckhoven, V., Huys, G., Vancanneyt, M., Vael, C., Klare, I., Romond, M.-B., Entenza, J. M., Moreillon, P., Wind, R. D., Knol, J., Wiertz, E., Pot, B., Vaughan, E. E., Kahlmeter, G. & Goossen, H. (2008) Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project. **19** (2) 102–114.

Vargha, M., Takáts, Z., Konopka, A. & Nakatsu, C. H. (2006) Optimization of MALDI-TOF MS for strain level differentiation of *Arthrobacter* isolates. *Journal of Microbiological Methods,* **66** (3) 399-409.

Vaughan, E. E., Heilig, H. G., Ben-Amor, K. & de Vos, W. M. (2005) Diversity, vitality and activities of intestinal lactic acid bacteria and bifidobacteria assessed by molecular approaches. *FEMS Microbiology Reviews,* **29** (3) 477-490.

Ventura, M., Canchaya, C., Fitzgerald, G. F., Gupta, R. S. & van Sinderen, D. (2007a) Genomics as a means to understand bacterial phylogeny and ecological adaptation: the case of bifidobacteria. *Antonie Van Leeuwenhoek,* **91** (4) 351-372.

Ventura, M., Canchaya, C., Meylan, V., Klaenhammer, T. R. & Zink, R. (2003) Analysis, characterization, and loci of the *tuf* genes in *Lactobacillus* and *Bifidobacterium* species and their direct application for species identification. *Applied and Environmental Microbiology,* **69** (11) 6908-6922.

Ventura, M., O'Connell-Motherway, M., Leahy, S., Moreno-Munoz, J. A., Fitzgerald, G. F. & van Sinderen, D. (2007b) From bacterial genome to functionality; case bifidobacteria. *International Journal of Food Microbiology,* **120** (1–2) 2-12.

Ventura, M., O'Flaherty, S., Claesson, M. J., Turroni, F., Klaenhammer, T. R., van Sinderen, D. & O'Toole, P. W. (2009a) Genome-scale analyses of health-promoting bacteria: probiogenomics. *Nature Reviews Microbiology,* **7** (1) 61-71.

Ventura, M., Turroni, F. & van Sinderen, D. (2012) Probiogenomics as a tool to obtain genetic insights into adaptation of probiotic bacteria to the human gut. *Bioengineered Bugs,* **3** (2) 73-79.

Ventura, M., Turroni, F., Zomer, A., Foroni, E., Giubellini, V., Bottacini, F., Canchaya, C., Claesson, M. J., He, F., Mantzourani, M., Mulas, L., Ferrarini, A., Gao, B., Delledonne, M., Henrissat, B., Coutinho, P., Oggioni, M., Gupta, R. S., Zhang, Z., Beighton, D., Fitzgerald, G. F., O'Toole, P. W. & van Sinderen, D. (2009b) The *Bifidobacterium dentium* Bd1 genome sequence reflects its genetic adaptation to the human oral cavity. *PLoS Genetics,* **5** (12) e1000785.

Ventura, M., van Sinderen, D., Fitzgerald, G. & Zink, R. (2004) Insights into the taxonomy, genetics and physiology of bifidobacteria. *Antonie Van Leeuwenhoek,* **86** (3) 205-223.

Ventura, M. & Zink, R. (2002a) Rapid identification, differentiation, and proposed new taxonomic classification of *Bifidobacterium lactis*. *Applied and Environmental Microbiology,* **68** (12) 6429-6434.

Ventura, M. & Zink, R. (2002b) Specific identification and molecular typing analysis of *Lactobacillus johnsonii* by using PCR-based methods and pulsed-field gel electrophoresis. *FEMS Microbiology Letters,* **217** (2) 141-154.

Vincent, D., Roy, D., Mondou, F. & Déry, C. (1998) Characterization of bifidobacteria by random DNA amplification. *International Journal of Food Microbiology,* **43** (3) 185-193.

Wall, R., Hussey, S. G., Ryan, C. A., O'Neill, M., Fitzgerald, G., Stanton, C. & Ross, R. P. (2007) Presence of two *Lactobacillus* and *Bifidobacterium* probiotic strains in the neonatal ileum. *ISME Journal,* **2** (1) 83-91.

Walter, J. (2008) Ecological role of lactobacilli in the gastrointestinal tract: Implications for fundamental and biomedical research. *Applied and Environmental Microbiology,* **74** (16) 4985-4996.

Walter, J., Tannock, G. W., Tilsala-Timisjarvi, A., Rodtong, S., Loach, D. M., Munro, K. & Alatossava, T. (2000) Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Applied and Environmental Microbiology,* **66** (1) 297-303.

Wang, K.-Y., Li, S.-N., Liu, C.-S., Perng, D.-S., Su, Y.-C., Wu, D.-C., Jan, C.-M., Lai, C.-H., Wang, T.-N. & Wang, W.-M. (2004) Effects of ingesting *Lactobacillus*- and *Bifidobacterium*-containing yogurt in subjects with colonized *Helicobacter pylori*. *The American Journal of Clinical Nutrition,* **80** (3) 737-741.

Wei, Y.-X., Zhang, Z.-Y., Liu, C., Zhu, Y.-Z., Zhu, Y.-Q., Zheng, H., Zhao, G.-P., Wang, S. & Guo, X.-K. (2010) Complete genome sequence of *Bifidobacterium longum* JDM301. *Journal of Bacteriology,* **192** (15) 4076-4077.

Williams, E. A., Stimpson, J., Wang, D., Plummer, S., Garaiova, I., Barker, M. E. & Corfe, B. M. (2009) Clinical trial: a multistrain probiotic preparation significantly reduces symptoms of irritable bowel syndrome in a double-blind placebo-controlled study. *Alimentary Pharmacology & Therapeutics,* **29** (1) 97-103.

Williams, T., Andrzejewski, D., Lay, J. & Musser, S. (2003) Experimental factors affecting the quality and reproducibility of MALDI TOF mass spectra obtained from whole bacteria cells. *Journal of the American Society for Mass Spectrometry,* **14** (4) 342-351.

Yamamoto, S. & Harayama, S. (1996) Phylogenetic analysis of *Acinetobacter* strains based on the nucleotide sequences of *gyrB* genes and on the amino acid sequences of their products. *International Journal of Systematic and Evolutionary Microbiology,* **46** (2) 506-511.

Yeung, P. S. M., Kitts, C. L., Cano, R., Tong, P. S. & Sanders, M. E. (2004) Application of genotypic and phenotypic analyses to commercial probiotic strain identity and relatedness. *Journal of Applied Microbiology,* **97** (5) 1095-1104.

Yeung, P. S. M., Sanders, M. E., Kitts, C. L., Cano, R. & Tong, P. S. (2002) Species-specific identification of commercial probiotic strains. *Journal of Dairy Science,* **85** (5) 1039-1051.

Yildirim, Z., Winters, D. K. & Johnson, M. G. (1999) Purification, amino acid sequence and mode of action of bifidocin B produced by *Bifidobacterium bifidum* NCFB 1454. *Journal of Applied Microbiology,* **86** (1) 45-54.

Yu, D. S., Jeong, H., Lee, D.-H., Kwon, S.-K., Song, J. Y., Kim, B. K., Park, M.-S., Ji, G. E., Oh, T. K. & Kim, J. F. (2012) Complete genome sequence of the probiotic bacterium *Bifidobacterium bifidum* strain BGN4. *Journal of Bacteriology,* **194** (17) 4757-4758.

Yutin, N., Puigbò, P., Koonin, E. V. & Wolf, Y. I. (2012) Phylogenomics of prokaryotic ribosomal proteins. *PLoS ONE,* **7** (5) e36972.

Zerbino, D. R. (2010) Using the Velvet *de novo* assembler for short-read sequencing technologies. *Current Protocols in Bioinformatics,* **Chapter 11** Unit 11 5.

Zhu, B., Xiao, D., Zhang, H., Zhang, Y., Gao, Y., Xu, L., Lv, J., Wang, Y., Zhang, J. & Shao, Z. (2013) MALDI-TOF MS distinctly differentiates nontypable *Haemophilus influenzae* from *Haemophilus haemolyticus*. *PLoS ONE,* **8** (2) e56139.

Zhu, L., Li, W. & Dong, X. (2003) Species identification of genus *Bifidobacterium* based on partial *hsp60* gene sequences and proposal of *Bifidobacterium thermacidophilum* subsp. *porcinum* subsp. nov. *International Journal of Systematic and Evolutionary Microbiology,* **53** (5) 1619-1623.

Zhu, Y., Zhang, Y. & Li, Y. (2009) Understanding the industrial application potential of lactic acid bacteria through genomics. *Applied Microbiology and Biotechnology,* **83** (4) 597-610.

Zhurina, D., Zomer, A., Gleinser, M., Brancaccio, V. F., Auchter, M., Waidmann, M. S., Westermann, C., van Sinderen, D. & Riedel, C. U. (2011) Complete genome sequence of *Bifidobacterium bifidum* S17. *Journal of Bacteriology,* **193** (1) 301-302.

9. APPENDICES

9.1. APPENDIX ONE

BULL, M. J., MARCHESI, J. R., VANDAMME, P., PLUMMER, S. & MAHENTHIRALINGAM, E. 2012. Minimum taxonomic criteria for bacterial genome sequence depositions and announcements. *Journal of Microbiological Methods,* 89 **(1)** 18-21.

Journal of Microbiological Methods 89 (2012) 18–21

Contents lists available at SciVerse ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Minimum taxonomic criteria for bacterial genome sequence depositions and announcements

Matthew J. Bull [a], Julian R. Marchesi [a], Peter Vandamme [b], Sue Plummer [c], Eshwar Mahenthiralingam [a,*]

[a] Organisms and Environment Division, Cardiff School of Biosciences, Cardiff University, Main Building, Museum Avenue, Cardiff, CF10 3AT, UK
[b] Laboratorium voor Microbiologie, Universiteit Gent, K. L. Ledeganckstraat 35, B-9000 Gent, Belgium
[c] Obsidian Research Ltd., Unit 2 Christchurch Road, Baglan Industrial Park, Port Talbot, Wales, SA12 7BZ, UK

ARTICLE INFO

Article history:
Received 2 February 2012
Accepted 11 February 2012
Available online 16 February 2012

Keywords:
Bacterial genomes
Taxonomy
Phylogenetics
Nomenclature

ABSTRACT

Multiple bioinformatic methods are available to analyse the information encoded within the complete genome sequence of a bacterium and accurately assign its species status or nearest phylogenetic neighbour. However, it is clear that even now in what is the third decade of bacterial genomics, taxonomically incorrect genome sequence depositions are still being made. We outline a simple scheme of bioinformatic analysis and a set of minimum criteria that should be applied to all bacterial genomic data to ensure that they are accurately assigned to the species or genus level prior to database deposition. To illustrate the utility of the bioinformatic workflow, we analysed the recently deposited genome sequence of *Lactobacillus acidophilus* 30SC and demonstrated that this DNA was in fact derived from a strain of *Lactobacillus amylovorus*. Using these methods researchers can ensure that the taxonomic accuracy of genome sequence depositions is maintained within the ever increasing nucleic acid datasets.

1. Introduction

The genomic revolution has impacted multiple fields of microbiology facilitating the application of DNA sequence-based analyses to numerous questions in ecology, infection and industry (Medini et al., 2008). Bacterial genome sequence information is highly accessible and with minimal training researchers that are not specialists in a given field can use it to develop a wealth of molecular tools such as strain or species-specific PCR markers. In doing so however, researchers are heavily reliant on the accurate deposition of data associated with genome sequences and the genetic databases. Although the taxonomy of many bacterial groups continues to change, the use of correct nomenclature for bacterial species is part of a fundamental language that allows microbiologists to communicate with each other and across other disciplines. Hence, when the species nomenclature associated with a bacterial genome sequence is incorrect it can have broad implications and impact on a multitude of fields. In 2008, (Field et al., 2008) recognised the need for minimum descriptive criteria for genomes and metagenomes. They provided examples of data deposition records listing multiple criteria that included taxonomic status as a leading descriptor (Field et al., 2008). However, correctly assigning taxonomic status to an organism's genome is generally left to the research group submitting the sequence. With the current quantity of sequence data being deposited, it is difficult for the DNA databases to further analyse the sequences and ensure that the taxonomic status of a genome is correct.

The availability of a complete bacterial genome sequence facilitates the application of several bioinformatic analyses to enable the source organism to be assigned to formally classified species or phylogenetic groups (Coenye et al., 2005). For all newly determined bacterial genomes it should therefore be relatively straightforward to systematically classify the bacterium from which the genome originates. However, the deposition of genome sequences assigned to the wrong systematic nomenclature may still occur if the sequences are not carefully analysed in a taxonomic context. Oh et al. (2011) recently deposited a genome sequence for *Lactobacillus acidophilus* 30SC, a bacterial isolate recovered from swine gut. The taxonomy of the genus *Lactobacillus* has changed considerably in recent years rendering biochemical or phenotypic analysis alone unable to permit accurate species identification for several constituent groups. For the existing *Lactobacillus* species however, DNA sequence-based methods can facilitate their accurate assignment at the species level (Naser et al., 2007). By examination of the *L. acidophilus* 30SC genome using just two defining characteristics, its full length 16S rRNA gene phylogeny and two conserved protein-encoding gene phylogenies, we were able to demonstrate that the genome sequence in fact must have originated from a strain of *Lactobacillus amylovorus*. To reduce the potential for such misclassification in future, we outline a simple bioinformatic analysis scheme and a minimum set of taxonomic criteria that should be applied to bacterial genomes before their formal deposition and announcement to the microbiology community.

* Corresponding author at: Cardiff School of Biosciences, Cardiff University, Room 0.11E Main Building, Museum Avenue, Cardiff, CF10 3AT, UK. Tel.: +44 29 20875875; fax: +44 29 20874305.
E-mail address: MahenthiralingamE@cardiff.ac.uk (E. Mahenthiralingam).

0167-7012/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2012.02.008

2. Materials and methods

2.1. L. acidophilus 30SC gene sequences

Full-length sequences of the 16S rRNA, *gyrB* and *pheS* genes were downloaded from the 30SC genome (GenBank ID: CP002559).

2.2. 16S rRNA gene systematics

The 16S rRNA gene sequence of *L. acidophilus* 30SC was compared to the Ribosomal Database Project II (RDP II; http://rdp.cme.msu.edu) databases using the sequence match (SeqMatch) tool to facilitate identification (Cole et al., 2009). The SeqMatch tool allowed the identification and subsequent acquisition of 16S rRNA gene sequences of 20 type strains that were most closely related to the 16S rRNA gene of *L. acidophilus* 30SC. These were imported into MEGA5 (Tamura et al., 2011), aligned and trimmed. MEGA5 was also used to construct a neighbour-joining phylogeny.

2.3. Protein-coding gene phylogenies

The gyrase B subunit gene, *gyrB*, sequences were acquired using the Functional Gene pipeline and repository (FunGene; http://fungene.cme.msu.edu); *gyrB* sequences from *Lactobacillus* type strains with 16S rRNA genes most closely related to *L. acidophilus* 30SC were specifically selected. Sequences of the phenylalanyl-tRNA synthase alpha subunit gene, *pheS*, were drawn from a study by (Naser et al., 2007) and downloaded from Genbank. The *gyrB* and *pheS* were imported into MEGA5 and analysed in the same way as the 16S rRNA gene sequences.

3. Results

3.1. A simple bioinformatic workflow for classification of bacterial genomes

A bioinformatic scheme to facilitate the accurate taxonomic identification of a bacterial genome was developed (Fig. 1). The workflow facilitated the assignment of genus- or species-level nomenclature in the case of known species, while for bacteria belonging to novel taxonomic groups it can be used to define the nearest phylogenetic neighbour. Since multiple bioinformatic analyses may be used to assign the taxonomy of a bacterial genome (Coenye et al., 2005), a simplified workflow dependent on just two phylogenetic criteria was selected. The scheme can be easily applied by researchers not necessarily skilled in bioinformatic analyses and despite its simplicity is sufficient to provide accurate resolution of a genome to a known species or taxonomic group.

The first analytical criterion analysed the 16S rRNA gene sequence of a genome (Fig. 1) as the most fundamental genetic tool available for bacterial taxonomic classification (Stackebrandt and Goebel, 1994). Bacterial 16S rRNA gene sequences can be rapidly compared at the Ribosomal Database Project II (RDP II; http://rdp.cme.msu.edu) or other curated databases and the search criteria limited to only identify closely related sequences obtained from well classified type strains. If related species type strains are not available for a given genome sequence, the related 16S rRNA gene sequences from the RDP II output may be searched for reference strains or well characterised sequences for uncultured microorganisms; these organisms can then be identified as the nearest defined phylogenetic neighbours of the genome sequence (Fig. 1).

The second taxonomic criterion applied in the bioinformatic workflow was to analyse the sequence of protein coding genes from the genome and compare them to homologs encoded within the related species/phylogenetic groups revealed by the initial 16S rRNA gene analysis (Fig. 1). Analysis of protein-coding genes such as *recA* (Eisen, 1995) or *gyrB* (Yamamoto and Harayama, 1996), has been shown to be highly effective in assigning the taxonomy of species with particularly conserved 16S rRNA gene sequences. However, since protein-coding gene phylogenies may not always be congruent with the 16S rRNA gene for many bacterial species due to the lower taxonomic resolution of the latter ribosomal gene and the possibility of recombination and lateral gene transfer (Lukjancenko et al., 2011), we suggest two or more protein-coding genes should be examined to bring a finer resolution to the taxonomic placement of a genome (Fig. 1).

In addition to using the latter databases to compare the 16S rRNA gene and selected protein-coding gene, phylogenetic trees of both taxonomic markers should also be constructed using the sequences from the most closely related species (Fig. 1). This step will ensure that an accurate evolutionary placement of genome can be made and avoid assigning relatedness based on the percentage match of DNA sequences. Examination of the 16S rRNA gene and protein-coding gene phylogenies should also be made to ensure they are consistent in

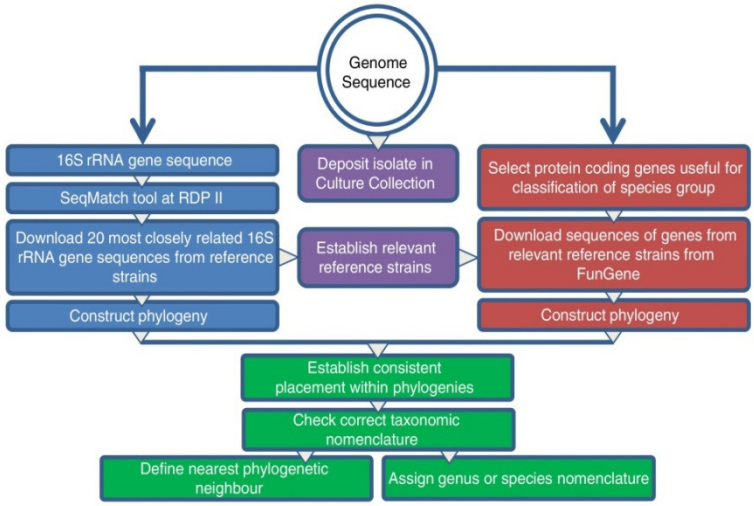


Fig. 1. Bioinformatic work flow for assignment of taxonomic status to a bacterial genome. An analysis scheme based primarily on analysis of the 16S rRNA gene with additional analysis of at least two protein coding genes is illustrated. The phylogeny of the latter genes should be compared for a given bacterial genome, and then the correct taxonomic nomenclature or nearest phylogenetic neighbour assigned. If the genome is representative of a cultured bacterial species, it should also be deposited in a recognised culture collection. The bioinformatic tools are available from the databases described in the Materials and Methods.

their assignment of the nearest match to the genome. On the basis of these combined analyses the correct up-to-date bacterial nomenclature can be obtained from the List of Prokaryotic names with Standing in Nomenclature (LSPN; http://www.bacterio.cict.fr). If the genome does not match a validly named species in terms of its 16S rRNA or protein-coding gene analyses, its nearest well characterised phylogenetic neighbour should be provided (Fig. 1).

3.2. Application of the bioinformatic workflow to the L. acidophilus 30SC genome

After deposition of the *L. acidophilus* 30SC genome, we performed a genome comparison to that of *L. acidophilus* NCFM, a well characterised probiotic strain of this species (Altermann et al., 2005), and observed that the two genomes were not closely related (data not shown). We therefore applied the bioinformatic analysis scheme and minimum criteria (Fig. 1) to *L. acidophilus* 30SC genome to clarify its taxonomic assignment. Use of the SeqMatch tool at the RDP II demonstrated that the *L. acidophilus* 30SC 16S rRNA gene was most closely related to *L. amylovorus* sequences. After downloading and phylogenetically analysing the full length 16S rRNA genes for 20 type strains of the most closely related *Lactobacillus* species, the resulting tree also demonstrated that *L. acidophilus* 30SC sequence was most similar to that of *L. amylovorus* LMG 9496^T (Fig. 2, panel A; 99.8% identity). The 16S rRNA gene for the *L. acidophilus*-type strain, LMG 9433^T, placed in a completely distinct phylogenetic cluster (Fig. 2, panel A; 98.2% identity).

Since the taxonomy of genus *Lactobacillus* has been heavily revised in recent years by the description of numerous new species and because the discriminatory power of the 16S rRNA gene for differentiation of its constituent taxa is limited for several clusters of species, we searched the available literature to identify protein-coding genes which were useful for species identification. The *gyrB* gene had been used as part of a multilocus sequence typing (MLST) scheme for *Lactobacillus plantarum* strains (de Las Rivas et al., 2006). We therefore used the FunGene database to compare the *gyrB* from the *L. acidophilus* 30SC genome to the *gyrB* genes available for type strains of those *Lactobacillus* species that were most closely related by analysis of the 16S rRNA gene (Fig. 2, panel A). The *gyrB* phylogeny demonstrated that the *L. acidophilus* 30SC *gyrB* sequence was most similar to that of *L. amylovorus* LMG 9496^T (Fig. 2, panel B; 99.8% identity). The *gyrB* sequence of the *L. acidophilus* type strain, LMG 9433^T, clustered separately from that of strain 30SC (Fig. 2, panel A) and was considerably less similar (88.1% identity).

Although the *gyrB* analysis was consistent with the 16S rRNA gene assignment, in order to avoid over reliance on a single-protein gene we searched the literature for an additional protein-coding gene useful for *Lactobacillus* systematics. Naser et al. (2007) had examined the *pheS* gene and demonstrated that it offered a discriminatory means of species identification within the genus *Lactobacillus*. The *pheS* gene of the *L. acidophilus* 30SC genome was 99.1% similar to that of *L. amylovorus* and phylogenetically clustered with this species (data not shown); it was not closely related to the *L. acidophilus pheS* sequence (88.7% identity). Overall, even though the *gyrB* (Fig. 2, panel A) and *pheS* (data not shown) phylogenetic trees were not absolutely congruent with 16S rRNA gene phylogeny across all the *Lactobacillus* species examined, the respective *L. acidophilus* 30SC sequences were consistently placed adjacent *L. amylovorus* as the nearest phylogenetic neighbour and indicating this species is the most likely taxonomic group to which the 30SC isolate belonged.

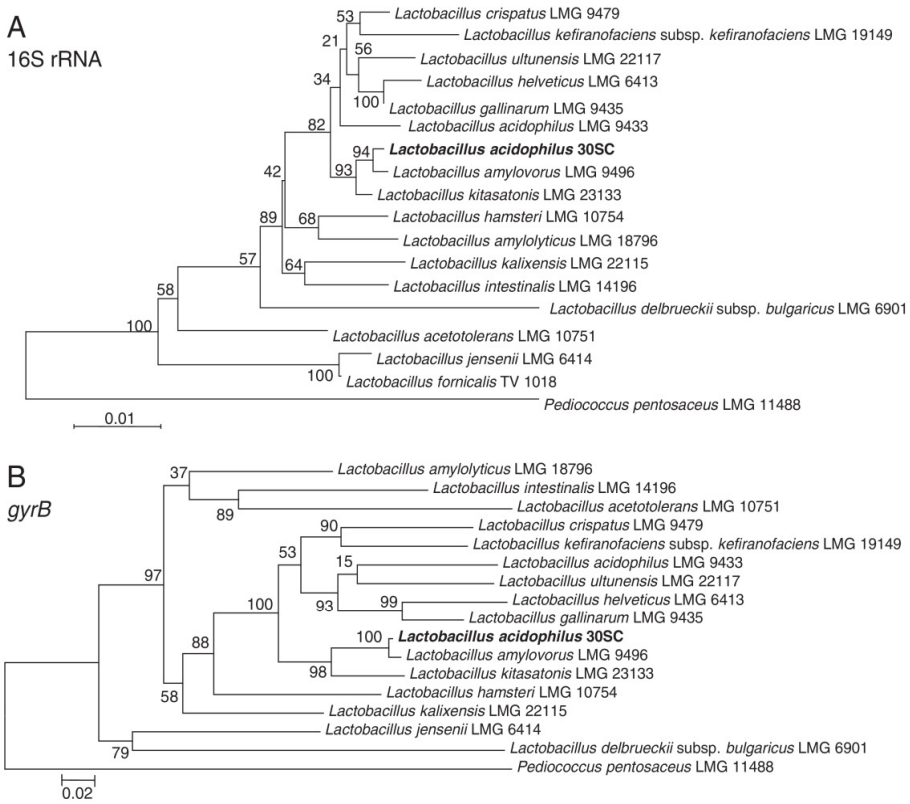


Fig. 2. Phylogenetic trees of *Lactobacilli* related to *L. acidophilus* 30SC. Phylogenetic analysis of aligned 16S rRNA (panel A) and *gyrB* (panel B) genes from representative *Lactobacillus* reference strains classified as most closely related (similarity scores >0.949) to the *L. acidophilus* 30SC (indicated in bold font) genes is shown. The trees for each gene were rooted with the corresponding sequence from *Pediococcus pentosaceus* LMG 11488; the genetic distance scale and bootstrap values are indicated.

4. Discussion

With the continued improvement and innovation in technology, particularly the advent of next-generation sequencers, researchers have unprecedented access to nucleotide sequence data. There is no doubt that the massive expansion of DNA sequence datasets has considerably advanced the study of life sciences, however, there is also a feeling that our ability to collect sequence data far surpasses our ability and power to analyse it (Brenner, 2010). In addition, as more sequence analysis tools are developed to enable large scale data mining, the outputs from these analyses may have less value if the original sequence data inputs are poorly characterised at source. Here we illustrate an example of how such oversights are still occurring in genomic microbiology, with the taxonomically incorrect deposition and announcement of the *L. acidophilus* 30SC genome (Oh et al., 2011). Using a straightforward analysis based on two minimal criteria, assignment via the 16S rRNA gene and protein-coding gene phylogenies, we have clearly demonstrated that the *L. acidophilus* 30SC genome most likely derives from a strain of *L. amylovorus* (Fig. 2).

Many disciplines rely heavily on taxonomic nomenclature to provide a common language that can be understood by both specialist researchers and also extend into wider public understanding. Taxonomy is particularly important in microbiology where there is such an extensive diversity of organisms that a microbial commons and systematic guidelines for nomenclature are absolutely vital for advancement of the discipline (Moore et al., 2010). Like many microorganisms, the lactic acid bacteria, within which *L. amylovorus* and *L. acidophilus* reside, are of considerable ecological, clinical and commercial interest (Pfeiler and Klaenhammer, 2007). The widespread use of these bacteria as probiotics and diary starter cultures forms a multi-million dollar industry, with basic researchers, industry and regulatory agencies demanding much clearer definitions of *Lactobacillus* strains. The incorrect deposition of the strain 30SC genome as a representative of the species *L. acidophilus* may potentially have had significant future ramifications, especially since genomes for these bacteria are now being obtained by non-specialist researchers and commercial groups.

In summary, we suggest the need for a systematic review of the way genome sequence data is deposited and propose a simple, minimum-standard system for characterisation of new bacterial genome sequences prior to their announcement. We have proposed an analytical scheme which is straightforward and for the most part can be performed using publicly available databases and software to compare the 16S rRNA gene and at least two protein-coding genes from a given genome. For researchers skilled in bioinformatic analyses, this scheme could easily be expanded to include analyses of multiple protein coding genes such as those used in MLST schemes (de Las Rivas et al., 2006), the average nucleotide identity of shared genes or even whole genome phylogenies (Konstantinidis and Tiedje, 2005). In addition, we also suggest that if the genome sequence is for an easily cultured microorganism, that the corresponding strain is deposited in a recognised International Depository Authority culture collection and hence can be easily analysed by the research community (Fig. 2); this will add considerable future value to a microbial genome sequence. We hope that our illustration of genome misclassification and a simple bioinformatic workflow to avoid it will increase the consistency of future genome sequence taxonomy. This will ensure that users of these incredibly valuable genomic datasets, particularly those who are not specialists in the field, can be confident in the identity of a deposited sequence.

Acknowledgements

M.B. is acknowledges funding from the Biotechnology and Biological Sciences Research Council PhD funding (Doctoral Training Grant BB/F016557/1) with CASE sponsorship from Cultech Ltd., Baglan, Wales, UK.

References

Altermann, E., Russell, W.M., Azcarate-Peril, M.A., Barrangou, R., Buck, B.L., McAuliffe, O., Souther, N., Dobson, A., Duong, T., Callanan, M., Lick, S., Hamrick, A., Cano, R., Klaenhammer, T.R., 2005. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. Proc. Natl. Acad. Sci. U. S. A. 102, 3906–3912.

Brenner, S., 2010. Sequences and consequences. Philos. Trans. R. Soc. B 365, 207–212.

Coenye, T., Gevers, D., Van de Peer, Y., Vandamme, P., Swings, J., 2005. Towards a prokaryotic genomic taxonomy. FEMS Microbiol. Rev. 29, 147–167.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M., 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37, D141–D145.

de Las Rivas, B., Marcobal, A., Munoz, R., 2006. Development of a multilocus sequence typing method for analysis of *Lactobacillus plantarum* strains. Microbiology 152, 85–93.

Eisen, J.A., 1995. The RecA protein as a model molecule for molecular systematic studies of bacteria: comparison of trees of RecAs and 16S rRNAs from the same species. J. Mol. Evol. 41, 1105–1123.

Field, D., Garrity, G., Gray, T., Morrison, N., Selengut, J., Sterk, P., Tatusova, T., Thomson, N., Allen, M.J., Angiuoli, S.V., Ashburner, M., Axelrod, N., Baldauf, S., Ballard, S., Boore, J., Cochrane, G., Cole, J., Dawyndt, P., De Vos, P., DePamphilis, C., Edwards, R., Faruque, N., Feldman, R., Gilbert, J., Gilna, P., Glockner, F.O., Goldstein, P., Guralnick, R., Haft, D., Hancock, D., Hermjakob, H., Hertz-Fowler, C., Hugenholtz, P., Joint, I., Kagan, L., Kane, M., Kennedy, J., Kowalchuk, G., Kottmann, R., Kolker, E., Kravitz, S., Kyrpides, N., Leebens-Mack, J., Lewis, S.E., Li, K., Lister, A.L., Lord, P., Maltsev, N., Markowitz, V., Martiny, J., Methe, B., Mizrachi, I., Moxon, R., Nelson, K., Parkhill, J., Proctor, L., White, O., Sansone, S.A., Spiers, A., Stevens, R., Swift, P., Taylor, C., Tateno, Y., Tett, A., Turner, S., Ussery, D., Vaughan, B., Ward, N., Whetzel, T., San, Gil I., Wilson, G., Wipat, A., 2008. The minimum information about a genome sequence (MIGS) specification. Nat. Biotechnol. 26, 541–547.

Konstantinidis, K.T., Tiedje, J.M., 2005. Genomic insights that advance the species definition for prokaryotes. Proc. Natl. Acad. Sci. U. S. A. 102, 2567–2572.

Lukjancenko, O., Ussery, D.W., Wassenaar, T.M., 2011. Comparative genomics of *Bifidobacterium*, *Lactobacillus* and related probiotic genera. Microb. Ecol. doi:10.1007/s00248-011-9948-y.

Medini, D., Serruto, D., Parkhill, J., Relman, D.A., Donati, C., Moxon, R., Falkow, S., Rappuoli, R., 2008. Microbiology in the post-genomic era. Nat. Rev. Microbiol. 6, 419–430.

Moore, E.R., Mihaylova, S.A., Vandamme, P., Krichevsky, M.I., Dijkshoorn, L., 2010. Microbial systematics and taxonomy: relevance for a microbial commons. Res. Microbiol. 161, 430–438.

Naser, S.M., Dawyndt, P., Hoste, B., Gevers, D., Vandemeulebroecke, K., Cleenwerck, I., Vancanneyt, M., Swings, J., 2007. Identification of lactobacilli by *pheS* and *rpoA* gene sequence analyses. Int. J. Syst. Evol. Microbiol. 57, 2777–2789.

Oh, S., Roh, H., Ko, H.J., Kim, S., Kim, K.H., Lee, S.E., Chang, I.S., Kim, S., Choi, I.G., 2011. Complete genome sequencing of *Lactobacillus acidophilus* 30SC, isolated from swine intestine. J. Bacteriol. 193, 2882–2883.

Pfeiler, E.A., Klaenhammer, T.R., 2007. The genomics of lactic acid bacteria. Trends Microbiol. 15, 546–553.

Stackebrandt, E., Goebel, B.M., 1994. Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44, 846–849.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

Yamamoto, S., Harayama, S., 1996. Phylogenetic analysis of *Acinetobacter* strains based on the nucleotide sequences of *gyrB* genes and on the amino acid sequences of their products. Int. J. Syst. Bacteriol. 46, 506–511.

9.2. APPENDIX TWO

BAXTER, C. G., RAUTEMAA, R., JONES, A. M., WEBB, A. K., BULL, M., MAHENTHIRALINGAM, E. & DENNING, D. W. 2013. Intravenous antibiotics reduce the presence of *Aspergillus* in adult cystic fibrosis sputum. *Thorax,* 68 **(7)** 652-657.

ORIGINAL ARTICLE

Intravenous antibiotics reduce the presence of *Aspergillus* in adult cystic fibrosis sputum

Caroline G Baxter,[1,2] Riina Rautemaa,[1,2] Andrew M Jones,[3] A Kevin Webb,[3] Matthew Bull,[4] Eshwar Mahenthiralingam,[4] David W Denning[1,2]

► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/thoraxjnl-2012-202412).

[1]The National Aspergillosis Centre, University Hospital of South Manchester, Manchester, UK
[2]Manchester Academic Health Science Centre, University of Manchester, Manchester, UK
[3]Manchester Adult Cystic Fibrosis Centre, University Hospital of South Manchester, Manchester, UK
[4]Organisms and Environment Division, Cardiff School of Biosciences, Cardiff University, Cardiff, South Glamorgan, UK

Correspondence to
Dr Caroline G Baxter, The National Aspergillosis Centre 2nd Floor Education and Research Centre, University Hospital of South Manchester, Southmoor Road, Manchester M23 9LT, UK; caroline.baxter@manchester.ac.uk

Received 12 July 2012
Revised 21 February 2013
Accepted 22 February 2013
Published Online First 19 March 2013

To cite: Baxter CG, Rautemaa R, Jones AM, *et al*. *Thorax* 2013;**68**:652–657.

ABSTRACT

Background *Pseudomonas aeruginosa* and *Aspergillus fumigatus* frequently co-colonise the airways of patients with cystic fibrosis (CF). This study aimed to assess the impact of short-term administration of intravenous antipseudomonal antibiotics during CF exacerbations on the presence of *Aspergillus*.

Methods Pre- and post-antibiotic sputum samples from 26 adult patients with CF and chronic *Pseudomonas* colonisation were analysed for the presence of *Aspergillus* by fungal culture, real-time PCR and galactomannan antigen (GM). Lung function (forced expiratory volume in 1 s and forced vital capacity % predicted) and blood levels of total IgE, specific *A fumigatus* IgE and specific *A fumigatus* IgG were measured at the start and end of antibiotics. Respiratory viral real-time PCR and bacterial community profiling using ribosomal intergenic spacer analysis (RISA) were performed to estimate concurrent changes in the lung microbiome.

Results *Aspergillus* PCR and GM were more sensitive than culture in detecting *Aspergillus* species (culture 8%, GM 31%, PCR 77%). There was a significant decline in the presence of *Aspergillus*, measured both by PCR and GM index, following antibacterial therapy (PCR: median increase in crossing threshold 1.7 (IQR 0.5–3.8), p<0.001; GM: median fall in GM index 0.7 (IQR 0.4–1.6), p=0.016). All patients improved clinically with a significant increase in lung function (p<0.0001). RISA community analysis showed large changes in bacterial community similarity in 67% of patients following antibiotics. Viral RT-PCR demonstrated the presence of a concurrent respiratory virus in 27% of patients.

Conclusions Intravenous antibiotics targeting *Pseudomonas* during CF pulmonary exacerbations have a negative impact on the presence of *Aspergillus* in sputum samples.

Key messages

What is the key question?
- Does the treatment of cystic fibrosis (CF) pulmonary exacerbations with antipseudomonal antibiotics affect the presence of *Aspergillus* in the sputum?

What is the bottom line?
- Short-course intravenous antibiotics targeting *Pseudomonas aeruginosa* reduce the presence of *Aspergillus*.

Why read on?
- An understanding of the interactions between these two important organisms within CF lungs could alter future therapeutic strategies and improve prognosis.

BACKGROUND

Recurrent pulmonary exacerbations are a predominant feature for many patients with cystic fibrosis (CF). Traditionally, treatment is with oral or intravenous antibiotics targeting bacteria grown from sputum culture. However, the CF lung microbiome is a complex environment consisting of many different bacteria, viruses and fungi, many of which may be contributing to pulmonary exacerbations. Molecular techniques to identify these organisms have demonstrated a much wider diversity of organisms than described by standard culture.[1 2]

The most common bacterium isolated from the sputum of adult patients with CF is *Pseudomonas aeruginosa*, which chronically colonises CF airways in up to 75% of adult patients.[3] The propensity for chronic infection is aided by biofilm formation driven by quorum sensing.[4] Biofilm formation is also thought to be a feature of *Aspergillus fumigatus* colonisation, the most prevalent filamentous fungus causing disease in CF.[5 6] *A fumigatus* is cultured from the sputum of 12–57% of patients with CF, but significantly higher rates of detection have been demonstrated by real-time PCR (RT-PCR).[7 8] The interaction between these two common organisms and their biofilms is a recent topic of interest, with studies showing that *P aeruginosa* inhibits *A fumigatus* growth in vitro, possibly due to secretion of small diffusible molecules.[9 10]

The short-term use of intravenous antipseudomonal antibiotics to treat pulmonary exacerbations is known to reduce *P aeruginosa* biomass transiently, but the concurrent effect on the presence of *A fumigatus* has not been studied.[11] However, long-term antibiotics (both oral and nebulised) used routinely to reduce pulmonary exacerbations and improve lung function have been linked to greater rates of *A fumigatus* colonisation, the mechanism and clinical impact of which is unknown.[12 13] While bacterial diversity appears to correlate with the use of long-term antibiotics, short-term antibiotics used for pulmonary exacerbations for 14–21 days have relatively little impact on diversity but do reduce overall bacterial biomass.[11] Any additional effect that these antibiotics have on organisms other than bacteria is largely unknown.

Furthermore, the agonist and antagonist relationships created between organisms during treatment are abundant but poorly understood.[14 15]

Determining the interactions between these two important organisms may result in new therapeutic strategies and improve prognosis for patients with CF. This study aimed to establish whether intravenous antibiotic therapy, targeting *P aeruginosa* during CF pulmonary exacerbations, affects the presence of *Aspergillus* determined by real-time PCR and galactomannan (GM) assay. Secondary aims were to estimate changing bacterial community profiles using ribosomal intergenic spacer analysis (RISA) and to assess the prevalence of concurrent viral respiratory infections.

METHODS

Study design and patient selection

This was a prospective observational cohort study. Patients were enrolled into the study from the Manchester Adult Cystic Fibrosis Centre, UK; all gave written informed consent to participate. Inclusion criteria included age ≥18 years, confirmed diagnosis of CF by genetic testing and/or sweat testing, chronic pulmonary *P aeruginosa* colonisation (determined by recurrent positive sputum cultures for >1 year) and recent onset pulmonary exacerbation leading to a decline in health status and lung function. The first 30 patients who met the inclusion criteria between November 2010 and March 2011 were recruited to the study. Patients were seen initially by CF specialist clinicians independent of the study and assessed with regard to their need for intravenous antibiotics and their suitability for outpatient treatment. Clinical samples were collected on day 1, immediately prior to antibiotic commencement (pre-antibiotics), and on day 14, following the last dose of antibiotics (post-antibiotics). Patients administered their own antibiotics at home for 14 days and then attended an end of treatment outpatient appointment. Tobramycin drug levels were monitored to assess toxicity and compliance.

Demographics and lung function

Baseline data were collected from patient records at enrollment and included demographic details, CF comorbidities, prior sputum microbiology and inhaled/oral medical treatments. Lung function (forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC)) was performed pre- and post-antibiotics by experienced technicians according to European Respiratory Society guidelines.[16]

Sputum collection

Patients produced two non-induced sputum samples pre-antibiotics and one sample post-antibiotics. Samples were refrigerated at ±4°C and processed within 24 h of collection. One of the two pre-antibiotic sputum samples was used for fungal culture, *Aspergillus* PCR and GM detection and the other sample was used for respiratory viral RT-PCR.

Sputum fungal culture

An equal volume of Sputasol (Oxoid, Basingstoke, UK) was added to the sputum sample and culture performed according to the Health Protection Agency National Standards Method BSOP 57 but modified to plate 10 µl rather than 1 µl of sputum onto three Sabouraud dextrose agar plates (SABC, Oxoid).[17] Sputum samples then underwent further homogenisation by sonication, as described previously, and culture was repeated.[8] Plates were incubated at 25°C, 37°C and 45°C for 72 h with daily inspection to record growth and colony forming units (CFUs). Fungal colonies were identified by microscopy.

Galactomannan

The Platelia *Aspergillus* enzyme immunoassay (Bio-Rad, Marnes-La-Coquette, France) was used to detect GM. Three hundred microlitres of homogenised sputum was processed in accordance with the manufacturer's instructions for serum samples and an optical density index of ≥0.5 was considered positive. Our group has demonstrated this assay to have an intra-assay coefficient of variation (CV) of 5% by simultaneously testing two aliquots of sputum from 20 patients with CF and an inter-assay CV of 9% when testing 12 samples each day over 5 days (C G Baxter, 2012, unpublished).

Aspergillus PCR

Fungal DNA was extracted from 3 ml of the remaining homogenised sputum sample using the commercial fungal DNA extraction kit MycXtra (Myconostica, UK) according to the manufacturer's instructions. This kit removes inhibiting substances such as dornase α and inhaled antibiotics and then uses bead beating to release DNA. *Aspergillus* DNA was detected using the commercial RT-PCR assay MycAssay Aspergillus (Myconostica), targeting a portion of the 18S ribosomal gene, on a SmartCycler RT-PCR instrument (Cepheid, California, USA); 10 µl of DNA template was used in 25 µl reactions. The manufacturer's instructions were followed in the processing of all DNA extractions except that suggested cut-off values were disregarded and the limit of blank, a crossing threshold (Ct) value of <38, was considered positive. This DNA extraction and RT-PCR assay has good reproducibility in CF sputum: our group has demonstrated an intra-assay CV of 1.5% when 10 homogenised sputum samples were split, DNA extracted and RT-PCR performed and an inter-assay CV of 1.1% when 40 extracted DNA samples were run through the RT-PCR assay on two occasions (C G Baxter, 2012, unpublished).

Ribosomal intergenic spacer analysis (RISA)

Following *Aspergillus* PCR, the remainder of the extracted DNA was used for bacterial diversity profiling by RISA. Intergenic spacer sizes vary between different bacterial species. Briefly, 20 ng of total sputum DNA (quantified using a NanoDrop 1000 spectrophotometer) and the RISA primers (1406F, 5′-TGYACACACCGCCCGT-3′ and 23R, 5′-GGGT TBCCCCATTCRG-3′; each at a final concentration of 10 pmol/µl in the reaction) were combined in a 25 µl PCR reaction using standard reagents (Qiagen, Crawley, UK) and amplified as described elsewhere.[18] The amplified intergenic spacer regions were then separated on an Agilent 2100 BioAnalyser (Agilent, Woking, UK) using the DNA 7500 microfluidics kit as described elsewhere.[19] The resulting bacterial diversity profiles were then analysed using GelCompar II (Applied Maths, Gent, Belgium) and before/after percentage similarity was calculated.

Viral PCR

The method and results of viral PCR can be found in the online data supplement (S1).

Serology

A blood sample was taken from each patient pre- and post-antibiotics. Each sample was tested for total IgE (tIgE), specific *A fumigatus* IgE (sIgE) and specific *A fumigatus* IgG (sIgG) using the ImmunoCap assay (Phadia, Uppsala, Sweden).

Statistics

SPSS V.16 (Chicago, USA) was used to analyse all results. The results were non-parametric and were compared using the Wilcoxon signed rank test and the Mann–Whitney U test.

RESULTS

Baseline demographics

Thirty patients gave consent to participate; 26 completed the study and four failed to attend their post-antibiotics appointment. The baseline demographic and clinical details are shown in table 1. The patients received dual intravenous antibiotics for 14 days; all patients received tobramycin and, in addition, 11 received ceftazidime, 11 meropenem, two piperacillin/tazobactam (Tazocin) and two aztreonam.

Sputum culture and PCR

Fifty-two sputum samples (pre- and post-antibiotic samples from 26 patients) were cultured. Routine fungal culture following Sputasol homogenisation showed no growth in 24 samples and yeast in 28 samples, whereas culture after additional sonication showed no growth in 18 samples, yeast in 32 samples and *A fumigatus* in two samples. The two samples culturing *A fumigatus* were both pre-antibiotic samples. Yeast identification was not performed.

Yeast was present in 16 patient samples pre-antibiotics and 16 post-antibiotics, but four patients became negative for yeast while four became positive. The four patients who became negative had very low numbers of CFUs (<4) pre-antibiotics. For the 16 patients with yeast cultured post-antibiotics, there was a significant increase in CFUs (post-sonication counts) after antibiotics (median CFU pre-antibiotics 4 (IQR 5–66), median CFU post-antibiotics 16 (IQR 4–163), Wilcoxon signed rank test $Z=-3.47$, $p<0.001$, $r=0.61$).

Twenty of the 26 patients (77%) had a positive *Aspergillus* PCR pre-antibiotics, of which 15 remained positive post-antibiotics. Six patients were PCR negative on both samples.

Table 1 Patient baseline clinical and demographic details: data are expressed and number (%) or mean±SD

Baseline clinical characteristics	n=26
Age (years)	25±8
Male	14 (54%)
F508 del homozygous	15 (58%)
BMI (kg/m^2)	21±3
CFRD	8 (31%)
Pancreatic insufficiency	25 (96%)
Inhaled steroids	21 (81%)
Long-term azithromycin	25 (96%)
Long-term nebulised antibiotics	25 (96%)
Triazole therapy >3 months prior to enrolment	2 (8%)
Chronic *Pseudomonas*	26 (100%)
Chronic *Burkholderia* spp	1 (4%)
Chronic MSSA	9 (35%)
Chronic MRSA	0 (0%)
NTM	1 (4%)
FEV_1% predicted	42±15
FVC % predicted	59±17

BMI, body mass index; CFRD, cystic fibrosis-related diabetes; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*; NTM, non-tuberculous mycobacteria.

There was a statistically significant increase in the PCR Ct value (indicating less DNA) between pre- and post-antibiotic sputum samples (median increase in Ct 1.7 (IQR 0.5–3.8), Wilcoxon signed rank test $Z=3.8$, $p<0.001$, $r=0.52$; table 2). Applying the standard curve of genomic concentrations against Ct values developed from serum samples, there was a median reduction in *Aspergillus* genomes of 91 (IQR 26–460) post-antibiotics (Wilcoxon signed rank test $Z=-3.12$, $p=0.002$).[20] This standard curve compares well with manufacturer data of extraction from spore solutions and with our data of extraction efficiency from CF sputum.[8 21] There were no correlations between antibiotic regime and PCR results.

Galactomannan

GM was positive in eight patients (31%) pre-antibiotics and in six patients (23%) post-antibiotics. Three patients converted from positive to negative while one converted from negative to positive. Seventeen patients remained GM negative in both sputum samples (table 2). There was a significant fall in GM index for the eight pre-antibiotic positive patients (median fall in GM index 0.7 (IQR 0.4–1.6)), (Wilcoxon signed rank test $Z=-2.42$, $p=0.016$, $r=0.5$).

A comparison of *Aspergillus* PCR with GM index showed that 15 patients were PCR positive on both samples, five of whom remained GM positive while 10 remained GM negative; five patients changed from PCR positive to negative, three of whom became GM negative and two were GM negative on both samples; six patients were PCR negative on both samples, five were also GM negative on both samples while one was GM positive after antibiotic therapy (table 2).

Ribosomal intergenic spacer analysis (RISA)

Total bacterial community profiling by RISA was only performed for the first six patients enrolled due to financial and time constraints (figure 1). A large change in community similarity following intravenous antibiotics was seen for four of the six patients. Pre-antibiotic and post-antibiotic profile similarities were 90%, 89%, 76% and 59%. Two patients showed little alteration in response to treatment (95% similarities). There was no correlation between degree of change in community similarity and changes in *Aspergillus* PCR. Although community similarity changed, actual diversity of bacteria did not alter.

Serology

Three patients had a tIgE >500 kIU/l, 14 patients had a sIgE ≥class 2 (0.7 kUa/l) and 20 patients had a sIgG >40 mg/l. Although all parameters fell with treatment, there was no statistically significant change in any parameter (table 3). However, patient numbers were small, meaning that the study was underpowered to detect small differences. Subanalysis of patients showed that those with a fall in *Aspergillus* DNA (rise in Ct value) or a fall in both *Aspergillus* DNA and GM index similarly had no significant changes in immunological parameters (table 3). However, there was a significant difference between baseline sIgG levels in those with positive PCR and GM pre-antibiotics (n=8) compared with those with negative PCR and GM (n=6) (median 118 mg/l (IQR 84–145) and 59 mg/l (IQR 43–83), respectively). Similarly, the fall in sIgG was significantly greater in those with positive PCR and GM pre-antibiotics (median fall 15 mg/l (IQR 7–23) and −4 mg/l (IQR 10–11.5), respectively; Mann–Whitney U test $Z=-2.39$, $p=0.013$). These differences were not seen for tIgE or sIgE.

Table 2 *Aspergillus* PCR and GM pre- and post-antibiotics

Patient	*Aspergillus* PCR Ct 1	*Aspergillus* PCR Ct 2	Change in *Aspergillus* PCR Ct	GM index 1	GM index 2	Change in GM index
1	36.3	37.8	1.5	7.13	2.48	−4.65
2	32.5	36.2	3.7	9.38	6.04	−3.34
3	32.4	32.9	0.5	2.97	0.86	−2.11
4	33.5	33.9	0.4	8.83	4.14	−4.69
5	35.0	36.3	1.3	0.73	0.70	−0.03
6	32.3	32.7	0.4	0.22	0.00	−0.22
7	33.1	36.2	3.1	0.00	0.12	0.12
8	30.4	33.3	2.9	0.00	0.00	0
9	31.3	33.3	2.0	0.19	0.16	−0.03
10	31.1	33.1	2.0	0.03	0.00	−0.03
11	31.3	33.6	2.3	0.30	0.19	−0.11
12	34.3	33.7	−0.6	0.00	0.00	0
13	30.8	34.9	4.1	0.00	0.00	0
14	35.4	36.6	1.2	0.06	0.00	−0.06
15	33.5	35.4	2.2	0.29	0.25	−0.04
16	30.4	38.0	7.6	2.92	0.27	−2.65
17	32.4	38.0	5.6	9.20	0.00	−9.20
18	33.8	38.0	4.2	1.28	0.12	−1.16
19	31.7	38.0	6.3	0.00	0.02	0.02
20	33.4	38.0	4.6	0.02	0.00	−0.02
21	38.0	38.0	0	0.45	10.53	10.08
22	38.0	38.0	0	0.00	0.00	0
23	38.0	38.0	0	0.25	0.39	0.13
24	38.0	38.0	0	0.49	0.39	−0.10
25	38.0	38.0	0	0.01	0.00	−0.01
26	38.0	38.0	0	0.00	0.01	0.01

Ct, crossing threshold; GM, galactomannan.

Lung function

Patients receiving intravenous antibiotic therapy demonstrated an improvement in both FEV_1 ($Z=-4.29$, $p<0.001$, $r=0.60$) and FVC ($Z=-4.46$, $p<0.001$), $r=0.62$; table 4). The rise in FEV_1 was greater for patients with positive *Aspergillus* PCR at the start of treatment than for those with negative PCR (Mann–Whitney U test $Z=-2.02$, $p=0.046$, $r=0.40$).

DISCUSSION

There is growing evidence for the wide microbial diversity within CF airways, but the dynamic interspecies communications within communities has only just begun to be investigated.[15] This study has shown a significant reduction in *Aspergillus* species, measured both by PCR and GM index, following antibacterial therapy targeting *P aeruginosa* colonisation. *P aeruginosa* colony counts were not performed in this study but previous studies have confirmed a significant reduction in colony counts with antipseudomonal antibiotics.[11 22]

The observed reduction in *Aspergillus* species with *Pseudomonas* treatment was unexpected as previous studies have suggested an increased prevalence of *A fumigatus* colonisation with the use of both oral and nebulised antibiotics.[12 13] Furthermore, in vitro studies have suggested that *P aeruginosa* inhibits *A fumigatus* growth and biofilm formation by secretion of small carbon chain molecules and the phenazine pyocyanin.[10 23] Thus, it was expected that a reduction in *P aeruginosa*

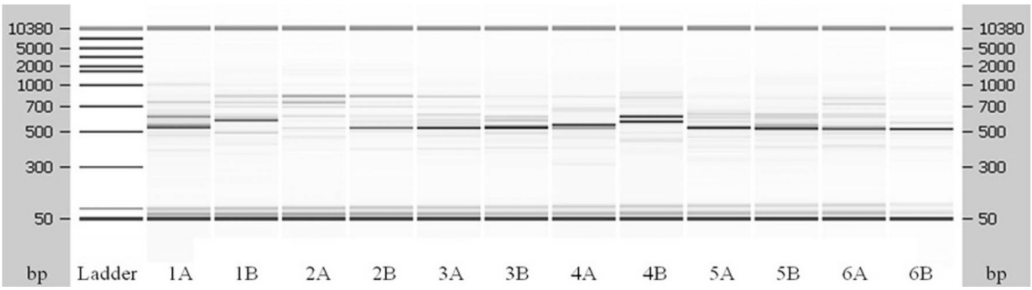


Figure 1 Ribosomal intergenic spacer analysis (RISA) profiles comparing community similarity of sputum samples from six patients (A) pre- and (B) post-intravenous antibiotics. The ladder provides a reference to estimate the size of RISA PCR products. Each lane shows the total bacterial population from each sputum sample. The change in community similarity for the six paired profiles shows: 1A–B=76%, 2A–B=90%, 3A–B=95%, 4A–B=59%, 5A–B=95% and 6A–B=89%.

Table 3 Changes in immunological parameters pre- and post-intravenous antibiotics

	Total IgE (kUI/l)		Wilcoxon signed rank test	*A fumigatus* specific IgE (kUa/l)		Wilcoxon signed rank test	*A fumigatus* specific IgG (mg/l)		Wilcoxon signed rank test
	Pre	Post		Pre	Post		Pre	Post	
All patients (n=26)	78 (28–230)	62 (25–220)	Z=−0.47 p=0.64	1.5 (0.0–8.9)	1.3 (0–7.7)	Z=−0.73 p=0.47	73 (50–112)	63 (51–100)	Z=−1.82 p=0.07
Patients with a fall in *Aspergillus* DNA (n=19)	110 (28–250)	79 (31–290)	Z=−1.19 p=0.24	7.8 (0.0–10.6)	7.0 (0.0–8.1)	Z=−0.93 p=0.35	78 (65–113)	75 (55–102)	Z=−1.50 p=0.13
Patients with a fall in *Aspergillus* DNA and GM index (n=8)	104 (14–293)	61 (21–253)	Z=−1.68 p=0.09	0.6	0.6	Z=0.00 p=1.00	118 (84–145)	103 (72–143)	Z=−1.12 p=0.26

Values are shown as median (IQR).
GM, galactomannan.

may lead to an increase in *A fumigatus*. The reasons for the observed fall in *Aspergillus* are unclear. Unlike short-term treatment, long-term antibiotics reduce bacterial diversity which may select more favourable growth conditions and host immunological responses for *Aspergillus* colonisation.[11] The effect of short-term intravenous antibiotics on biofilms must also be considered. It is important to note that the inhibitory effects of *P aeruginosa* on *A fumigatus* were only found to be significant by Mowat *et al*[10] prior to biofilm formation, in keeping with the incomplete inhibition seen during the in vitro studies by Kerr *et al*.[9] *P aeruginosa* biofilms offer some protection against antibacterial effects, as do *A fumigatus* biofilms against antifungal susceptibility,[24 25] but biofilms can be disrupted by antibiotics such as macrolides.[26] Biofilm disruption may allow *P aeruginosa* to re-exert its inhibitory effects on *A fumigatus* growth. The interdependence between organisms for survival within the lung has not been studied, but it is also possible that *A fumigatus* may use *P aeruginosa* biofilms for host immune protection and favourable growth conditions meaning disruption would inhibit the presence of both organisms. These concepts are speculative and further research could have significant implications for CF therapeutic strategies and prognosis.

Total bacterial diversity and community profiling was only performed in this study for a very limited number of patients, which is a significant limitation of this aspect of the work. However, large changes in community similarity were seen in four of six patients. Total bacterial diversity may not change significantly, but the abundance of particular bacteria in each community does change as represented by the altering intensities of PCR bands (figure 1). This is supported by a recent study by Tunney *et al* which showed that changes in bacterial abundance for aerobes was greater than for anaerobes, with *P aeruginosa* being affected most when using antipseudomonal antibiotics.[11] These changes in community profile may also impact on the presence and growth of *Aspergillus* as bacteria other than *P aeruginosa* can inhibit fungal growth.[27] Future studies with greater patient numbers would clearly benefit from full bacteriological culture and more robust molecular analysis of changing bacterial populations during antibiotic therapy to improve our understanding of the dynamic parallel changes in fungal populations within this context.

Both *Aspergillus* PCR and GM were more sensitive than culture for the detection of *Aspergillus* species (culture 8%, GM 31%, PCR 77%). PCR detects both live and dead organisms along with dormant spores whereas GM is predominantly produced by hyphae in the logarithmic phase of growth and is a major component of the biofilm.[6 28] This may account for the differences in pre-antibiotic detection rates between these two tests as a large number of patients with CF are thought to have simple colonisation with inert or dead *A fumigatus* spores while fewer have active hyphal growth. This is also supported by the significant difference observed for specific *A fumigatus* IgG levels between patients with positive PCR and GM pre-antibiotics (n=8) and those without (n=6) (median sIgG 118 mg/l and 59 mg/l, respectively). The patients with positive PCR and GM pre-antibiotics also showed a trend towards a reduction in tIgE, sIgG, *Aspergillus* DNA and GM index post-antibiotics. It is not known how long antibody concentrations take to change significantly, but this study was conducted over a short time period and patient numbers were not powered to detect these serological changes.

One patient receiving piperacillin/tazobactam showed an increase in GM after antibiotics while remaining PCR negative. Intravenous antibiotics including piperacillin/tazobactam have been reported to lead to false positive results from serum GM analysis.[29] This is thought to be due to assay cross-reactivity with non-*Aspergillus* carbohydrate chains rather than contamination of products, and GM index levels are usually not very high. The rise in GM index in this case was high at 10.5 and no other samples suggested cross-reactivity. However, *Aspergillus* PCR was negative, in keeping with an alternative source of GM. GM is not specific to *Aspergillus* and can be found in other

Table 4 Changes in lung function pre- and post-antibiotics

	All patients (n=26)			Positive *Aspergillus* PCR (n=20)			Negative *Aspergillus* PCR (n=6)		
	Pre-antibiotics	Post-antibiotics	p Values	Pre-antibiotics	Post-antibiotics	p Values	Pre-antibiotics	Post-antibiotics	p Values
FEV_1% predicted	30 (22–36)	36 (26–50)	<0.001	33 (25–38)	39 (30–51)	<0.001	22 (21–33)	24 (22–43)	0.07
FVC % predicted	49 (35–59)	59 (44–69)	<0.001	52 (36–60)	63 (49–70)	<0.001	35 (31–47)	41 (36–64)	0.03

Values are shown as median (IQR).
Changes in lung function were measured using Wilcoxon signed rank tests.
FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity.

fungi and *Candida* cell walls. This patient did not grow any yeast or other fungi pre- or post-antibiotics, but the influence of oropharnygeal flora is a possible caveat in this study as separate oral sampling was not performed. Our previous research has indicated that the prevalence of both *A fumigatus* and *Candida* species from oral rinse samples are very low during non-exacerbation periods.[30] However, intravenous antibiotics may predispose to oral *Candida* infections which could potentially influence GM results post-antibiotics.

In summary, this study has shown value for both PCR and GM in monitoring *Aspergillus* concentrations and growth in CF sputum. Short-term intravenous antibiotics targeting *P aeruginosa* appear to have a negative impact on the presence of *Aspergillus*. The clinical impact of this observation requires further research.

Acknowledgements We would like to thank all the staff at the Manchester Adult Cystic Fibrosis Centre for their assistance in conducting this study, particularly William Flight for all his help with patient coordination.

Contributors CGB is guarantor of the study. CGB, RR, AMJ and DWD contributed to the study conception and design; CGB and EM contributed to the acquisition of data; CGB, MB, EM and DWD contributed to the interpretation and analysis of data; CGB drafted the manuscript and all authors revised the manuscript critically for important intellectual content and provided final approval of the version to be published.

Funding This study was supported by the National Commissioning Group, National Aspergillosis Centre, University Hospital of South Manchester, UK. MB is supported by a BBSRC Doctoral Training Grant (BB/F016557/1).

Competing interests No support was received from any organisation for the submitted work. Relevant financial activities outside the submitted work in the last 3 years include: CGB has received travel grants from Merck and Pfizer and has been paid for talks on behalf of Astellas; EM has received grants from the UK Cystic Fibrosis Trust, Technology Strategy Board and BBSRC; DWD acts as an advisor/consultant to Lab21 and T2Biosystems, has been paid for talks on behalf of Pfizer, Gilead and Astellas, holds founder shares in F2G Ltd and has received travel support from Astellas; RR, AMJ, KW and MB report no relevant financial activities outside the submitted work.

Ethics approval Ethics approval was obtained from the South Manchester Research Ethics Committee (07/Q1403/70).

Provenance and peer review Not commissioned; internally peer reviewed.

REFERENCES

1 Nagano Y, Elborn JS, Millar BC, *et al*. Comparison of techniques to examine the diversity of fungi in adult patients with cystic fibrosis. *Med Mycol* 2010;48:166–76 e1.
2 Bittar F, Rolain JM. Detection and accurate identification of new or emerging bacteria in cystic fibrosis patients. *Clin Microbiol Infect* 2010;16:809–20.
3 Valenza G, Tappe D, Turnwald D, *et al*. Prevalence and antimicrobial susceptibility of microorganisms isolated from sputa of patients with cystic fibrosis. *J Cyst Fibros* 2008;7:123–7.
4 Singh PK, Schaefer AL, Parsek MR, *et al*. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* 2000;407:762–4.
5 Muller FM, Seidler M, Beauvais A. *Aspergillus fumigatus* biofilms in the clinical setting. *Med Mycol* 2011;49(Suppl 1):S96–S100.
6 Loussert C, Schmitt C, Prevost MC, *et al*. In vivo biofilm composition of *Aspergillus fumigatus*. *Cell Microbiol* 2009;12:405–10.
7 Bakare N, Rickerts V, Bargon J, *et al*. Prevalence of *Aspergillus fumigatus* and other fungal species in the sputum of adult patients with cystic fibrosis. *Mycoses* 2003;46:19–23.
8 Baxter CG, Jones AM, Webb K, *et al*. Homogenisation of cystic fibrosis sputum by sonication—an essential step for *Aspergillus* PCR. *J Microbiol Methods* 2011;85:75–81.
9 Kerr JR. Suppression of fungal growth exhibited by *Pseudomonas aeruginosa*. *J Clin Microbiol* 1994;32:525–7.
10 Mowat E, Rajendran R, Williams C, *et al*. *Pseudomonas aeruginosa* and their small diffusible extracellular molecules inhibit *Aspergillus fumigatus* biofilm formation. *FEMS Microbiol Lett* 2010;313:96–102.
11 Tunney MM, Klem ER, Fodor AA, *et al*. Use of culture and molecular analysis to determine the effect of antibiotic treatment on microbial community diversity and abundance during exacerbation in patients with cystic fibrosis. *Thorax* 2011;66:579–84.
12 Jubin V, Ranque S, Stremler Le Bel N, *et al*. Risk factors for *Aspergillus* colonization and allergic bronchopulmonary aspergillosis in children with cystic fibrosis. *Pediatr Pulmonol* 2010;45:764–71.
13 Bargon J, Dauletbaev N, Kohler B, *et al*. Prophylactic antibiotic therapy is associated with an increased prevalence of *Aspergillus* colonization in adult cystic fibrosis patients. *Respir Med* 1999;93:835–8.
14 Han MK, Huang YJ, Lipuma JJ, *et al*. Significance of the microbiome in obstructive lung disease. *Thorax* 2012;67:456–63.
15 Harrison F. Microbial ecology of the cystic fibrosis lung. *Microbiology* 2007;153:917–23.
16 Miller MR, Hankinson J, Brusasco V, *et al*. Standardisation of spirometry. *Eur Respir J* 2005;26:319–38.
17 Health Protection Agency. UK Standards for Microbiology Investigations. Investigation of bronchoalveolar lavage, sputum and associated specimens; BSOP 57, 2008. http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1317132860548 (accessed 24 Nov 2012).
18 Borneman J, Triplett EW. Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Appl Environ Microbiol* 1997;63:2647–53.
19 White J, Gilbert J, Hill G, *et al*. Culture-independent analysis of bacterial fuel contamination provides insight into the level of concordance with the standard industry practice of aerobic cultivation. *Appl Environ Microbiol* 2011;77:4527–38.
20 White PL, Perry MD, Moody A, *et al*. Evaluation of analytical and preliminary clinical performance of Myconostica MycAssay Aspergillus when testing serum specimens for diagnosis of invasive Aspergillosis. *J Clin Microbiol* 2011;49:2169–74.
21 Myconostica. MycXtra DNA extraction—effective extraction of fungal DNA from clinical respiratory samples. http://myconostica.co.uk/mycxtra (accessed 24 Nov 2012).
22 Regelmann WE, Elliott GR, Warwick WJ, *et al*. Reduction of sputum *Pseudomonas aeruginosa* density by antibiotics improves lung function in cystic fibrosis more than do bronchodilators and chest physiotherapy alone. *Am Rev Respir Dis* 1990;141:914–21.
23 Kerr JR, Taylor GW, Rutman A, *et al*. *Pseudomonas aeruginosa* pyocyanin and 1-hydroxyphenazine inhibit fungal growth. *J Clin Pathol* 1999;52:385–7.
24 Kobayashi H. Airway biofilms: implications for pathogenesis and therapy of respiratory tract infections. *Treat Respir Med* 2005;4:241–53.
25 Seidler MJ, Salvenmoser S, Muller FM. *Aspergillus fumigatus* forms biofilms with reduced antifungal drug susceptibility on bronchial epithelial cells. *Antimicrob Agents Chemother* 2008;52:4130–6.
26 Lutz L, Pereira DC, Paiva RM, *et al*. Macrolides decrease the minimal inhibitory concentration of anti-pseudomonal agents against *Pseudomonas aeruginosa* from cystic fibrosis patients in biofilm. *BMC Microbiol* 2012;12:196.
27 Yadav V, Gupta J, Mandhan R, *et al*. Investigations on anti-Aspergillus properties of bacterial products. *Lett Appl Microbiol* 2005;41:309–14.
28 Hope WW, Petraitis V, Petraitiene R, *et al*. The initial 96 hours of invasive pulmonary aspergillosis: histopathology, comparative kinetics of galactomannan and (1->3) beta-d-glucan and consequences of delayed antifungal therapy. *Antimicrob Agents Chemother* 2010;54:4879–86.
29 Boonsarngsuk V, Niyompattama A, Teosirimongkol C, *et al*. False-positive serum and bronchoalveolar lavage *Aspergillus* galactomannan assays caused by different antibiotics. *Scand J Infect Dis* 2010;42:461–8.
30 Baxter CG, Moore CB, Jones AM, *et al*. IgE mediated immune responses and airway detection of *Aspergillus* and *Candida* in adult cystic fibrosis. *Chest*. Published Online First: 8 November 2012. doi:10.1378/chest.12–1363