An *in vitro* and *in vivo* assessment of *Lactobacillus plantarum* and lactulose as an intervention strategy against *S.* Typhimurium in pigs

A thesis submitted by

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For the degree of

Doctor of Philosophy

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Royal Holloway, University of London

2013

Dedication

"The right word may be effective, but no word was ever as effective as a rightly timed pause."

- Mark Twain

To my parents

Declaration of authorship

I Monika Anna Tchórzewska hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

Signed:

Date:

Abstract

S. Typhiumrium is a ubiquitous zoonotic pathogen, which remains a recognised human health risk factor in the food production chain. Following the withdrawal of antimicrobial growth promoters in animal feeds in the European Union, the enhancement of the host's resistance to enteropathogens by alternative control strategies has become essential. Probiotics are classified as live microbial feed supplements; often members of the normal flora and have been shown to improve gut health and further act as competitors against foodborne pathogens. Prebiotics are non-digestable in the upper gut, however fermented in the large intestine carbohydrates aimed to selectively stimulate beneficial bacteria and therefore selectively modulate gut microbiota.

Probiotics, prebiotic have been reported as successful in conferring protection against many pathogens. Therefore the aim of this project was the construction of an effective synbiotic combination of pre and probiotic that can be employed to control *Salmonella* colonisation in pigs.

The results demonstrated that lactulose promoted the growth of *L. plantarum* in the pure culture and in fermentations with the complex porcine microflora, resulting in an increase of short chain fatty acid (SCFA) concentrations. The presence of *L. plantarum* cell-free supernatant (CFS) conferred a strong inhibitory activity against *S.* Typhimurium growth. Moreover, *L. plantarum* together with the CFS reduced *S.* Typhimurium adherence and invasion to porcine epithelial cells. Inclusion of *L. plantarum* and lactulose in a batch culture system resulted in the overall gradual decrease in *Salmonella* numbers as evaluated by fluorescent *in situ* hybridisation (FISH), which was correlated with the increases in SCFA. Finally, an *in vivo* trial was undertaken using a pig infection model. The results generated from this *in vivo* study in pigs, confirmed the superior effect of the probiotic candidate *L. plantarum* in combination with lactulose in comparison to probiotic or prebiotic alone. Together with the higher lactobacilli counts in the synbiotic feed group, a reduced frequency of *Salmonella* shedding was observed.

Acknowledgments

Foremost, I would like to thank my supervisors Professor Roberto La Ragione and Professor Martin Woodward for giving me the opportunity to work on this project. I also wish to thank them for their continued encouragement, support and guidance throughout the project and for their invaluable constructive critique in preparing this thesis. Moreover, I would like to extend my thanks to my university supervisor Professor Peter Bramley.

I gratefully acknowledge the funding received towards this PhD from the Food Standards Agency, without which this project would not be possible.

I would like to thank Dr Adele Costabile for her help and guidance with the fluorescent *in situ* hybridisation technique and for her always welcoming approach. I wish to thank Dr Javier Salguero, from the AHVLA Histopathology department for his expertise and assistance with the *post-mortem* examination, during the *in vivo* trial.

I also extend my regards to the members of staff in the Cell and Tissue Culture Section and in the Animal Service Unit for their expertise and technical support.

I would like to thank to all my colleges at the Department of Bacteriology at the AHVLA for creating a stimulating and friendly environment. To Manal AbuOun, Wael Abdelrahman, Chistine Boinett for their support and friendship. A special thank you to Luke Mappley, for all the support and assistance provided, when most needed.

Most of all, I would like to thank my family, for their endless encouragement and support, especially during the final stages of this project.

Table of Contents

List of figures

List of tables

List of abbreviations

AFLP	Amplified fragment length polymorphism
AHVLA	Animal Health and Veterinary Laboratories Agency
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
ASP	Acid shock proteins
ATP	Adenosine triphosphate
ATR	Acid tolerance response
ATCC	American Type Culture Collection
BGA	Brilliant green agar
BLAST	Basic local alignment search tool
bp	Base pair
BPEX	British pig executive
BPW	Buffered peptone water
BSA	Bovine serum albumin
BSH	Bile Salt Hydrolase
CCP	Critical control points
CE	Competitive exclusion
CFS	Cell free supernatant
CFU	Colony forming unit
CO_2	Carbon dioxide
CTAB	Cetyltrimethylammonium bromide
DAPI	4′6-diamidino-2-phenylindole
DEFRA	Department for Environment, Food and Rural Affairs
DMEM	Dulbeco's modified eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dUTP	Deoxyuridine triphosphate
DTT	Dithiothreitol
EC	European Commission
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EPS	Expolysaccharide
EU	European Union
FCS	Foetal calf serum
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
FISH	Fluorescent *in situ* hybridisation
FOS	Fructooligosaccharide
FSA	Food Standards Agency
g	Gram
GALT	Gut-associated lymphoid tissue
GAP	GTPase-activating protein
GDP	Guanosine triphosphate
GEF	Guanine nucleotide exchamge factor
GIT	Gastrointestinal tract
GLU	Glucose

GOS	Galactooligosaccharide
GRAS	Generally regarded as safe
GTP	Guanosine diphosphate
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulphide
HACCP	Hazard Analysis Critical Control Point
HB	Hybridization buffer
HBSS	Hank's balanced salt solution
HE	Hematoxylin and eosin
HGT	Horizontal gene transfer
HIB	Heart infusion broth
HMO	Human milk oligosaccharides
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxide
Ig	Immunoglobulin
IPEC-J2	Porcine intestinal epithelial cell line
ITS	Iso-Sensitest
IVOC	*In vitro* organ culture
Kb	Kilobase pair
LAB	Lactic acid bacteria
LAC	Lactulose
LB-G	Lysogeny broth without glucose
LSM	LAB susceptibility medium
LTA	Lipoteichoic acid
M	Molar
MALDI	Matrix-assisted laser desorption/ionisation
mg	Milligram
MIC	Minimum inhibitory concantration
ml	Millilitre
MLST	Multilocus sequence typing
MLVA	Multilocus variable number of tandem repeats analysis
MLN	Mesenteric lymph node
MM	Minimal medium
mM	Millimolar
MOS	Mannan-oligosaccharide
MRS	De Man-Rogosa-Sharpe
MSC	Mucosal competitive exclusion
MSP	Main spectral projection
MSRV	Modified Semi-Solid Rappaport-Vassiliadis
MUCAP	4-methylumbelliferyl caprylate
NBF	Neutral buffered formalin
NCBI	National Centre for Biotechnology Information
NCTC	National Collection of Type Cultures
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PT	Phage type
rpm	Resolutions per minute

RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal ribonucleic acid
TTSS	Type three secretion system
UK	United Kingdom
WHO	World Health Organisation
v/v	volume/volume
w/v	veight/volume
ZAP	Zoonosis Action Plan
ZNCPig	Zoonoses National Control Program for *Salmonella* in pig meat
XLD	Xylose lysine deoxycholate agar
3D	Three-dimensional
RV	Rappaport-Vassiliadis medium
RWV	Rotating wall vesell
SBA	Sheep's blood agar
SCFA	Short chain fatty acids
SCAN	Scientific committee on animal nutrition
SCV	*Salmonella* containing vacuole
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
Sif	*Salmonella* induced filament
SPI	*Salmonella* pathogenicity island
TAE	Tris-acetate-EDTA
TCDA	Taurodeoxycholic acid
TE	Tris-EDTA
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TTSS	Type three secretion system
UK	United Kingdom
WHO	World Health Organisation
v/v	volume/volume
w/v	veight/volume
ZAP	Zoonosis Action Plan
ZNCPig	Zoonoses National Control Program for *Salmonella* in pig meat
XLD	Xylose lysine deoxycholate agar
3D	Three-dimensional

Chapter 1

Introduction

1.1 *Salmonella* - global zoonotic problem

Salmonellae are one of the major causes of food-borne disease worldwide (Adams and Moss, 2008). The global human health impact of non-typhoidal *Salmonella* is significant with estimated 93.8 million illnesses, of which approximately 80.3 million are food-borne (Majowicz *et al*., 2010). In the European Union (EU), salmonellosis is consistently the second most frequently reported zoonotic disease in humans (EFSA, 2012). In 2010 the most commonly reported *Salmonella* serovars were *S*. Enteritidis and *S*. Typhimurium accounting for around 80% of all confirmed human salmonellosis cases. Nevertheless, other serovars including Infantis, Newport, Virchow, Hadar, Mbandaka are prevalent (EFSA, 2012). *Salmonella* infections in humans and animals may lead to high morbidity and in some instances high mortality. Clinically, human salmonellosis is usually manifested by diarrhea, nausea, vomiting, intestinal cramping and mild fever. The severity of the symptoms differs, in general infections are self limited, but in very young and elderly salmonellosis can lead to severe dehydration and become life-threatening (Adams and Moss, 2008; Santos *et al*., 2001). Globally, is it estimated that 155,000 deaths occur due to non-typhoidal salmonellosis each year (Majowicz *et al*., 2010).

In 2010 in the EU the number of human illnesses due to salmonellosis decreased by 8.8% compared to the previous year with 99,020 in 2010 and 108,618 in 2009 (EFSA, 2012). It was concluded that the reduction of human salmonellosis is largely a consequence of implemented *Salmonella* control programs in chicken populations (Collard *et al*., 2008; EFSA, 2012). In the UK, the number of laboratory-confirmed human salmonellosis cases has declined with a total of 10,071 confirmed cases in 2009 and 9,685 in 2010 (DEFRA, 2009, 2010; EFSA, 2012). However, *S*. Enteritidis serovar remained the most commonly reported serovar whilst *S*. Typhimurium, the second

most commonly reported serovar, increased by 4.6% (DEFRA, 2010).

Salmonella infections result in significant economic losses and it has been reported that the total cost of all *Salmonella* infections in the EU, accounting also for premature deaths, are equivalent to nearly €600 million (Anonymous, 2010). Santos *et al*. (2011) reported that when the societal costs were estimated for *S*. Enteritidis and *S*. Typhimurium, the latter seem to be more expensive to treat. It has been concluded that cases of human salmonellosis caused by *S*. Enteritidis are associated with the consumption of contaminated eggs and poultry meat, whereas contaminated pig, poultry and bovine meat is linked to *S*. Typhimurium infections (EFSA, 2010, 2012). Total costs of *Salmonella* infections associated with pork in EU are estimated to be around €90 million (Anonymous, 2010). Considering the public health impact and economic burden and the fact that pork and pork products have been recognised as important source of human salmonellosis (Lo Fo Wong *et al*., 2002) it is important to reduce *Salmonella* levels in pigs and pork, thus reduce the risk of human salmonellosis due to pig origin.

1.2 *Salmonella* general characteristics

1.2.1 History and nomenclature

The genus *Salmonella* was named after the American veterinary pathologist; Dr Daniel Salmon and his colleague Dr Theobald Smith who first isolated the "hog cholera bacillus" from a pig in 1885 (Salmon and Smith, 1886) and which at the time was considered to be a cause of swine plague. Interestingly, these rod-shaped, Gram-negative bacteria were visualized beforehand by Eberth in 1880 in tissue sections from spleen and mesenteric lymph nodes of patients with typhoid fever and then isolated by Gaffky in 1884 (Grimont *et al*., 2000; Le Minor, 1994; Parry, 2006).

Historically, *Salmonella* species were classified on the basis of their epidemiology, host range, clinical manifestation, biochemical reactions and surface antigenic pattern. Since then, there have been many revisions and nomenclature changes in the classification of *Salmonella* genus (Euzeby, 1999; Ezaki *et al*., 2000a; Ezaki *et al*., 2000b; Le Minor and Popoff, 1987) and, as concluded by Brenner *et al*. (2000), standardization was considered necessary. Thus, the current concept distinguished two species which are *S. enterica* and *S. bongori*, the latter was formerly known as subspecies V (Reeves *et al*., 1989). Within *Salmonella enterica* six known

subspecies are listed; *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica* (Le Minor and Popoff, 1987; Reeves *et al*., 1989; Tindall *et al*., 2005).

Serological classification of *Salmonella* is based on the antibody interaction with the salmonellae lipopolisaccharides (O antigen), flagellar proteins (H antigen) and capsular polysaccharides (Vi antigen). To date all identified serovars and their antigenic formulae are listed in the White-Kaufmann-Le Minor Scheme (Grimont and Weill, 2007; Popoff *et al*., 2004; Switt *et al*., 2009). The *Salmonella* serotyping scheme, formerly known as Kauffmann-White Scheme is revised by the Institut Pasteur, WHO Collaborating Centre for Reference and Research (Popoff *et al*., 2001; Popoff *et al*., 2004). More than 2500 *Salmonella* serotypes are known, with the majority belonging to *S. enterica* subsp. *enterica* (Grimont and Weill, 2007) (Table 1.1). Some *Salmonella* serovars can be further subdivided into phage types (PT) and to date phage typing, is a frequently used method for epidemiological characterisation of pathogens (Baggesen *et al*., 2010).

Table 1.1 Number of serovars in each species and subspecies of *Salmonella* (Grimont and Weill, 2007).

Species and subspecies	**Number of serovars**
S. enterica	2557
subsp. *enterica*	1531
subsp. *salamae*	505
subsp. *arizonae*	99
subsp. *diarizonae*	336
subsp. *houtenae*	73
subsp. *indica*	13
S. bongori	22
Total	2579

1.2.2 Phenotypic characteristics

Salmonella are Gram-negative, catalase-positive, oxidase- negative, rod-shaped bacteria belonging to the family Enterobacteriaceae and phylum Proteobacteria. The majority of *Salmonella* strains are motile with peritrichous flagella, which can be encoded by two different flagellin genes on the bacterial chromosome (*fli*C and *flj*B) (Switt *et al*., 2009). Salmonellae are facultative anaerobes, ferment glucose and

produce hydrogen sulfide from thiosulphate. In general *Salmonella* strains are prototrophic and, as a result, they can grow easily in minimal medium with glucose as a sole carbon source, ammonia as a nitrogen source and mineral salts (Grimont *et al*., 2000). A small number of host-adapted *Salmonella* serovars are auxotrophs and require additional growth factors as they are not able to synthesize some essential vitamins and amino acids and therefore supplements including vitamins (thiamin, nicotinic acid), purines (adenine and guanine) and the amino acids (cystine, methionine, leucine, threonine, histidine, arginine, and aspartic acid) must be supplied in the medium to support their growth (Stokes and Bayne, 1958). For example, *S.* Gallinarum requires thiamine and *S.* Pullorum a presence of nicotinic acid, leucine, aspartic acid and cysteine (Stokes and Bayne, 1958).

Some phenotypic characteristics are often used for identification of bacteria, e.g. the production of hydrogen sulphide from thiosulphate or the ability to hydrolyse 4-methylumbelliferyl caprylate (MUCAP) leading to release of fluorescent umbelliferone (Olsson *et al*., 1991). Most *Salmonella* subspecies lack the ability to produce β-$_D$-galactosidase, which distinguishes them from other *Enterobacteriaceae* members such as *E. coli* (Kuhn *et al*., 1994). Moreover, as lactose, sucrose, salicin cannot be fermented by *Salmonella*, those are together with the pH indicators often included in the selective isolation media (Grimont *et al*., 2000).

1.2.3 *Salmonella* host range

Salmonella serotypes can be divided into host-restricted, host-adapted and un-restricted serotypes (Uzzau *et al*., 2000). The latter includes *S.* Enteritidis and *S.* Typhimurium and, in general, clinically those are associated with gastroenteritis which is most often self limiting (Rabsch *et al*., 2002; Uzzau *et al*., 2000). On the contrary, human host-restricted serotypes such as *S.* Typhi and *S.* Paratyphi cause severe systemic diseases (McClelland *et al*., 2004; Santander and Curtiss, 2010). Similarly, Abortusequi, Gallinarum, Typhisuis, Abortusovis, are almost completely restricted to equine, fowl, swine, ovines respectively (Alam *et al*., 2009; Pardon *et al*., 1988; Shivaprasad, 2000; Uzzau *et al*., 2000). Furthermore, the host-adapted group includes *S.* Dublin and *S.* Choleraesuis, which predominantly cause disease in cattle or pig with disease in other species, including human, possible (Bolton *et al*., 1999; Chiu *et al*., 2004; Uzzau *et al*., 2000). It is suggested that the development of the host adaptation was driven through multiple horizontal transfer events which resulted in new gene

combination, especially genes that belong to *Salmonella* pathogenicity islands, virulence plasmid, fimbrial operons, pseudogenes and lysogenic phages (Eswarappa *et al*., 2008; Kingsley and Baumler, 2000).

1.2.4 Transmission and persistence

Although the main transmission route is thought to be the faecal-oral route, it has been observed that tonsils and lungs are important sites for *Salmonella* invasion with intranasal transmission via dust and an aerosol is probable (Fedorka-Cray *et al*., 1995; Gray *et al*., 1995).

The ability of *Salmonella* to survive long-term outside the host results in environmental contamination and creates the possibility for the transmission of infection (Baloda *et al*., 2001; Winfield and Groisman, 2003). It has been demonstrated that *Salmonella* persist in the pigs, faecal samples, feed and the piggery environment for over 2 years (Baloda *et al*., 2001). Similarly, genetically indistinguishable serotypes of *Salmonella* in outdoor swine wallows were isolated for over 5 months (Callaway *et al*., 2005). Factors including temperature, oxygen, chemical and biological composition of the environment influence survival time (Semenov *et al*., 2009). The ability of *Salmonella* to persist in the environment raises the awareness of additional sources of contamination, which can be rodents, insects, contaminated feed and feedstuff. Moreover, this highlights the importance of environmental decontamination and focuses research on alternative control strategies.

1.2.5 Pathogenesis

1.2.5.1 *Salmonella* stress resistance and attachment

The clinical symptoms of gastroenteritis range from asymptomatic to severe diarrhoea. In order to colonise a host, bacteria must survive unfavorable conditions such as gastric acid, detergent effects of bile, decreasing oxygen supply, coexisting bacteria and immune responses (Rychlik and Barrow, 2005). *Salmonella* resist low pH by expressing an acid tolerance response (ATR) which can protect the cell down to pH3 (Foster, 2001). In response to acidic conditions *Salmonella* synthesize sets of proteins which confer a protective role and thus facilitate survival and replication (Foster, 1991; Foster and Hall, 1990; Wilmes-Riesenberg *et al*., 1996). The ATR of *S.* Typhimurium requires the synthesis of over 50 acid shock proteins (ASPs) and there

are at least two known ATR systems, one in logarithmic growth phase and the second in stationary phase (Wilmes-Riesenberg *et al*., 1996). *Salmonella* harbour numerous regulons allowing the adaptation to low pH, and it was demonstrated that two distinct tolerance systems for surviving the organic and inorganic acid stress exist (Bearson *et al*., 1998). RpoS and Fur, global regulatory proteins, are essential for surviving the organic acid stress, whereas partially redundant protection against inorganic acid stress is afforded by two RpoS and PhoPQ dependent systems (Bearson *et al*., 1998). The PhoP-PhoQ system has also been implicated in the regulation of *Salmonella* virulence genes (Bearson *et al*., 1998). In addition to PhoP-PhoQ system *Salmonella* virulence is regulated by the OmpR/EnvZ signal transduction system, associated with osmolarity-dependant regulation of OmpC and OmpF porins (Rychlik and Barrow, 2005).

Salmonella spp. express a range of fimbrial and non-fimbrial adhesins which facilitate site specific colonisation of host cells, therefore playing an important role in the host infection (Edwards *et al*., 2000). Several types of fimbriae associated with *Salmonella* may play a role in colonisation including type 1 fimbriae (Fim), plasmid encoded fimbriae (Pef), long polar fimbriae (Lpf) and thin aggregative fimbriae (Curli) (Darwin and Miller, 1999). Interestingly, Baumler *et al*. (1996b) demonstrated that *S*. Typhimurium fimbriae bind specifically receptors that are expressed on the particular cell type. This study supported the previously investigated concept that *S*. Typhimurium adhesion to the murine small intestine villous is mediated via Pef, whereas Lpf mediates adhesion to ileal Peyer's patches (Baumler and Heffron, 1995; Baumler *et al*., 1996a). Studies have shown that Fim are important in the site specific attachment of *Salmonella* to various eukaryotic cells (Darwin and Miller, 1999; Duguid *et al*., 1966). Ewen *et al*. (1997) demonstrated that these proteinaceous appendages contain mannose sensitive lectin, and this binds to the epithelial cells which express α-d-mannose receptors.

Salmonella also express flagella which confer motility and are thought to be a significant factor for pathogen attachment and invasion (Dibb-Fuller *et al*., 1999; Jones *et al*., 1992). Studies conducted by Allen-Vercoe and Woodward, 1999 demonstrated that flagella mutants were less capable of attaching to chicken tissues *in vitro* and proven to be less pathogenic *in vivo*. Flagella and especially chemotaxis enable *Salmonella* to respond to the various attractants and repellent gradients and was declared as essential for efficient induction in murine *Salmonella* – induced colitis (Stecher *et al*., 2004).

1.2.5.2 *Salmonella* invasion and diarrhoea

Once attached, the entry of *Salmonella* into a host cell requires synchronized action of numerous bacterial effector proteins (Galan and Zhou, 2000). The pathogen protein secretion systems are important for bacteria to induce disease and can be encoded by particular regions of pathogenicity islands. Those are large genomic regions (10-200kb) of DNA encoding virulence factors found in pathogenic strains and acquired by via horizontal gene transfer (Hacker and Kaper, 2000; Morgan, 2007).

Salmonella spp. harbours five main *Salmonella* pathogenicity islands (SPIs), namely, SPI-1, SPI-2, SPI-3, SPI-4 and SPI-5 although some 23 are now described (Hayward personal communication) (Eswarappa *et al*., 2008). SPI-1 is essential for *Salmonella* to invade the eukaryotic cell and induce the intestinal secretory and inflammatory responses, whereas SPI2 plays a role in systemic infections and intracellular accumulation of *Salmonella* and for its survival (Darwin and Miller, 1999; Morgan, 2007; Wallis and Galyov, 2000). The SPI encoded type three secretion system, TTSS, is one of the most complex protein secretion systems comprising over 20 proteins (Galan, 2001). The main function of this complex secretion system is delivery of bacterial proteins to the host cells (Galan and Collmer, 1999). The TTSS supramolecular structure named a “needle complex” consists of a numerous substructures and architecturally resembles a flagellar hook-basal body (Kubori *et al*., 1998; Kubori *et al*., 2000). The basal part of the “needle complex” consists of two pairs of rings attached to the inner and outer membranes of the bacterial cell which are connected with an inner rod. Several structural proteins of the “needle complex” have been recognised, PrgK, InvG, PrgH are known to form the basal part, the PrgJ is the putative inner rod protein and the needle portion is formed by a PrgI protein (Kubori *et al*., 2000; Sukhan *et al*., 2003). In addition, the InvA, SpaP, SpaQ, SpaR and SpaS proteins are a part of an export apparatus (Galan and Wolf-Watz, 2006).

The TTSS-1 system enables, through excellent synchronization between translocators and translocated effector proteins, the continuous delivery of circa thirteen effector proteins (Kubori *et al*., 2000; Main-Hester *et al*., 2008). Translocators, such as SipB, SipC and SipD are a vital group of proteins for translocation of the effector proteins into a host cells, nevertheless the full mechanism of this delivery has yet to be fully elucidated (Galan and Wolf-Watz, 2006; Galan and Zhou, 2000). Directly after the contact of *Salmonella* with the host cells, effectors including SopE, SopE2 (with function similar to SopE) and SopB are involved in the reversible

activation of the Rho family GTPases (Galyov *et al*., 1997; Hardt *et al*., 1998). The latter being the key regulators of the host cellular architecture are under control of guanine exchange factors (GEFs), which assist in GTP binding, and also the GTPase activating proteins (GAPs) which promotes GTP hydrolysis (Moon and Zheng, 2003; Patel and Galan, 2005). In *Salmonella* pathogenesis the exchange of inactive GDP for GTP active state of Cdc42 and Rac-1 cytoskeleton regulatory proteins is directly catalysed by SopE effector protein, functioning here as GEF (Hardt *et al*., 1998; Zhou *et al*., 1999). Moreover, the alternative, indirect activation exist, Cdc42, but no Rac-1 is mediated through SopB effector protein by modification of phosphoinositide phosphate (PIP) and inositol phosphate (IP) metabolism (Patel and Galan, 2005; Zhou *et al*., 2001). The activation of Rho family GTPases by pathogen effectors leads to actin cytoskeleton rearrangements and membrane ruffling through the involvement of host Arp2/3 complex along with bacterial SipA and SipC effector proteins, triggering *Salmonella* internalization (Hayward and Koronakis, 2002; McGhie *et al*., 2009). Interestingly, studies by Hanisch *et al*. (2010) demonstrated that while complex activator WAVE-2 of Arp2/3 is necessary for the formation of ruffles, the latter is not required for *Salmonella* entry. Following bacterial internalization, SptP effector protein is involved in reversing cytoskeletal changes by antagonizing GEF function and switching Cdc42 and Rac-1 proteins to the GDP-bound inactive state (Fu and Galan, 1999).

Once inside the host cell *Salmonella* survives and replicates in the *Salmonella*-containing vacuole (SCV). The early stage of SCV biogenesis is mediated via numerous *Salmonella* effector proteins of SPI-1 with subsequent involvement of SPI-2 TTSS (Steele-Mortimer, 2008). This second TTSS is activated under intracellular conditions as mentioned above and is necessary for *Salmonella* survival and proliferation (Brown *et al*., 2005; Hensel, 2000). SeeB, SseC and SseD proteins are required for translocation of effector proteins across the membrane of the vacuole, but in contrast to SPI-1 these do not posses effector functions (Waterman and Holden, 2003). Whereas proteins such as SipC, SseF, SseG, SifA, SspH1, SspH2, SseJ, SrfJ and PipB are translocated (Waterman and Holden, 2003). These effectors assist in the interference with endocytic trafficking, protection from reactive oxygen and reactive nitrogen species, formation of tubular aggregates of endosomal compartments (Sifs), cholesterol accumulation, induction of delayed macrophage apoptosis and assembly of intracellular actin (Kuhle *et al*., 2004; Miao *et al*., 2003; Steele-Mortimer, 2008;

Uchiya *et al*., 1999; Waterman and Holden, 2003).

The SCV migrates through the epithelial cell to the basolateral membrane and subsequently interacts with associated with Peyer's patches in the submucosal space macrophages (Ohl and Miller, 2001). In response to pathogen recognition, chemokine production generates neutrophil influx and transmigration into a lumen. It is suggested that interleukin-8, potent neutrophil chemokine is important in recruiting neutrophils to the submucosal space (Ohl and Miller, 2001). Loss of epithelial integrity is caused by neutrophil inflammation and is linked with necrosis of the mucosa which, in turn, leads to diarrhoea (Santos *et al*., 2003; Zhang *et al*., 2003).

1.3 *Salmonella* in pigs and pork

1.3.1 Clinical disease and pathology

Salmonella infections in pigs are associated with both broad-range and host adapted serotypes and characterised by diverse clinical symptoms (Fedorka-Cray *et al*., 2000). Host-adapted *S.* Choleraesuis causes a swine paratyphoid with clinical manifestations of enterocolitis and septicemia and is correlated with high mortality in young animals (Wilcock and Schwartz, 1992). Importantly, infection with this serotype is not limited to pigs only, but also systemic infections in human (Chiu *et al*., 2004).

Nevertheless, the main subject of this thesis is a ubiquitous serotype *S.* Typhimurium, to which pigs undoubtedly are predisposed among other hosts. It must be highlighted that although broad host range serotypes including *S.* Typhimurium could cause a disease as discussed in this section, in general those infections remain subclinical (Boyen *et al*., 2008a; Fedorka-Cray *et al*., 2000; Kranker *et al*., 2003). Importantly, the carriage of *Salmonella* in tonsils, intestines and the gut-associated lymphoid tissue (GALT) by asymptomatic carriers pose a risk to human health (Fedorka-Cray *et al*., 2000). It is when an appropriate pathogen population is reached that affects the virulence gene expression, the disease occurs (Lawley and Walker, 2013).

Clinical disease caused by *S.* Typhimurium, is linked with enterocolitis, characterised by high morbidity rates and with yellow, watery diarrhea, pyrexia, inappetence and lethargy (Fedorka-Cray *et al*., 2000; Wilcock and Schwartz, 1992). The recovery is usually quick, leading to asymptomatic carriage and intermittent

shedding of *Salmonella* in faeces (Nielsen *et al*., 1995; Verbrugghe *et al*., 2011; Wilcock and Olander, 1977; Wilcock and Schwartz, 1992). In pigs infected with *S*. Typhimurium macroscopic lesions found during necropsy are mainly located in the spiral colon, they constitute of localized or diffuse catarrhal colitis with hemorrhages and erosions, and can also form diffuse diptheresis (Wilcock, 1979; Wilcock *et al*., 1976). Similar but less apparent lesions are observed in the descending colon and rectum (Wilcock *et al*., 1976). Microscopically, in the colon the mild mucosal necrosis and mononuclear infiltration into the lamina propria is observed (Wilcock, 1979; Wilcock *et al*., 1976).

1.3.2 Diagnosis

As mentioned in the previous section, the non-typhoid *Salmonella* infections in pigs often results in mild or no clinical signs. Consequently, a range of diagnostic procedures is used to detect *Salmonella* in pigs and later during other stages of pork production. The isolation *Salmonella* from animal faeces consist of several steps. Those include pre-enrichment, selective enrichment and the use of selective plating media, which is dictated by factors such as, sub-lethal bacterial injury, low number of excreted bacteria and mixed sample population (Adams and Moss, 2008). The detection result often depends on the sample size, type of medium and incubation temperature (Davies *et al*., 2000; Funk *et al*., 2000). The EU approved method for the detection of *Salmonella* in food and animal feedstuffs ISO 6579:2002 (Annex D) consist of pre-enrichment of the sample in buffered peptone water (PBW), followed by selective enrichment in modified version of Rappaport-Vassiliadis (RV) enrichment medium (Vassiliadis, 1983) called Modified Semi-Solid Rappaport-Vassiliadis (MSRV) (Aspinall *et al*., 1992; De Smedt and Bolderdijk, 1987) and further plating onto a two selective agar plates (ISO, 2002). Agar media, used for plating out following enrichment contain selective agents such as bile salts or deoxycholate and/or brilliant green (Adams and Moss, 2008). Those include brilliant green agar (BGA) (Kauffmann, 1935), xylose lysine deoxycholate (XLD) agar and Rambach agar (Rambach, 1990), often used for isolation of *Salmonella*, particularly subspecies *enterica* (Carrique-Mas and Davies, 2008; Kuhn *et al*., 1994). At the farm level confirmation of *Salmonella* infection can sometimes be restricted due to duration and intermittent shedding (Ivanek *et al*., 2012; Wilcock and Schwartz, 1992). Serological testing of blood serum and meat juice using enzyme-linked immunosorbent assay

(ELISA) is therefore used to detect *Salmonella* antibodies and to asses sero-prevalence, particularly on farm when the whole herds are tested and at the abattoir level (Hill *et al*., 2008; Nielsen *et al*., 1995; Nielsen *et al*., 1998; Vico and Mainar-Jaime, 2011). The onset of the serological response following the infection could differ from weeks to months (Kranker *et al*., 2003; Nielsen *et al*., 1995) with resultant low serum IgG concentrations (Barrow, 2000), which could often be a limitation.

Various biochemical and serological methodology combined with further phenotypic and genotypic characterisation is used to fully identify disease aetiological agent, and it is particularly helpful during the common-source outbreaks studies (Threlfall and Frost, 1990; Wilcock and Schwartz, 1992). Testing based on the phenotypic characteristics such as serotyping, biotyping, phage typing, antibiotic susceptibility testing is used for the subdivision of *Salmonella* serovar. Further, genetic subdivision is granted with plasmid profiling, amplified fragment length polymorphism (AFLP), pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST), variable number of tandem repeat (VNTR) analysis and multilocus variable number of tandem repeats analysis (MLVA).

1.3.3 Risk factors associated with infection and transmission

Identification and re-evaluation of *Salmonella* associated risk factors in pork production is essential for implementation of suitable control strategies. One of the potential sources of *Salmonella* at the farm is animal feed that is now recognised as a significant source of infection (Davies and Wray, 1997; Fedorka-Cray *et al*., 1997b; Lo Fo Wong *et al*., 2002). Moreover, it should be regarded that risk of animal feed contamination can arise at feed mills, during transport and storage (Davies *et al*., 2004; Fedorka-Cray *et al*., 1997b; Linton *et al*., 1970). With regards to physical properties of the feed, it has been reported that feeding non-pelleted, coarsely ground meal could have a protective effect against infection and it is correlated with higher abundance in anaerobic microflora and increases in organic acids concentrations in the porcine gastrointestinal tract (Lo Fo Wong *et al*., 2002; Mikkelsen *et al*., 2004). Acidification of feed with organic acids is a recognised effective control measure (Davies *et al*., 2004). Transmission of *Salmonella* might also occur by way of mechanical vectors including other farm or domestic animals, birds, flies and rodents, hence their control is essential in order to prevent introduction of *Salmonella* (Davies and Wray, 1995; Liebana *et al*., 2003; Lo Fo Wong *et al*., 2002; Wang *et al*., 2011). Other on farm risk

factors are associated with the type of flooring and housing used and slatted floors have been linked with reduced *Salmonella* prevalence (Davies *et al*., 1997b; Letellier *et al*., 1999). Greater *Salmonella* shedding could be attributed to housing pigs in barns with open-flush gutters (Davies *et al*., 1997a). Oversized herds can create stressful environments in which immunity may be compromised; therefore intensively reared pigs may be more prone to infection. *Salmonella* express their virulence genes in presence of norepinephrine (Bearson and Bearson, 2008; Bearson *et al*., 2008) which is produced by stressed pigs (Rosochacki *et al*., 2000). Norepinephrine enhances *Salmonella* pathogenesis and leads to increased pathogen shedding and environmental contamination (Bearson and Bearson, 2008; Pullinger *et al*., 2010). The stress factor would also play a role during pig transport to the abattoir as well as the contaminated truck itself (Fedorka-Cray *et al*., 1994; Lo Fo Wong *et al*., 2002). Moreover, as demonstrated by Swanenburg *et al*. (2001a) keeping pigs in the lairage introduces a risk of *Salmonella* infection.

Salmonella has been identified at all levels of pork production, thus further carcass and pork product contamination can occur at the slaughterhouse, packing plant, processing and retail level (Lo Fo Wong *et al*., 2002). Multiple risk factors regarding *Salmonella* contamination and cross-contamination at abattoir-level have been identified (Swanenburg et al., 2001) and the need for adequate hygiene regimes highlighted (Botteldoorn *et al*., 2003). At that level of pork production, the critical control points (CCP) should be identified and regularly monitored in line with the Hazard Analysis Critical Control Point (HACCP) system to avoid carcass contamination (Lo Fo Wong *et al*., 2002).

1.3.4 Prevalence in pigs and pork

Contaminated with *Salmonella* pork is recognised as an important source of human gastroenteritis (Swanenburg *et al*., 2001) that has a significant economic impact linked to human health care and loss of work productivity (Fedorka-Cray *et al*., 1997a). The prevalence of *Salmonella* in fattening pigs at the point of slaughter was determined in a baseline survey carried out in 2006/2007 (Anonymous, 2010). In the EU, the prevalence in slaughter pigs infected with *Salmonella* in ileo-caecal lymph nodes was 10.3% and in the UK it was 21.2% with 13.8% attributable to *S.* Typhimurium, the most frequently isolated serovar at EU and national level. In the same survey in the UK, 13.5% of pig carcases were contaminated with *Salmonella* of

which 7.2% was due to *S*. Typhimurium (EFSA, 2008a). In 2008 another baseline survey on the prevalence of *Salmonella* in holdings with breeding pigs was carried out across the EU (EFSA, 2009) because of their potential role in disseminating *Salmonella*. The overall prevalence was 31.8% (*S*. Typhimurium 7.8% and *S*. Derby 8.9%). The prevalence of *Salmonella*-positive breeding holdings in the EU was 28.7% and in the UK was 52.2% with 19.4% prevalence attributable to *S*. Typhimurium. The results on prevalence of *Salmonella*-positive production holdings showed that in the EU prevalence was 33.3% and in the UK 44% with 9.9% prevalence attributable to *S*. Typhimurium (EFSA, 2009). Those baseline studies were aimed to provide important information with regards to setting *Salmonella* reduction targets and for future assessment of the impact that control programs might have.

1.3.5 The aim to reduce *Salmonella*

In response to emerging animal and zoonotic diseases and increasing international trade higher interest in veterinary surveillance systems has been reported (Stark *et al*., 2006). In June 2002 in response to the high incidence of *Salmonella* in pigs, the British Pig Executive (BPEX) in partnership with Food Standards Agency (FSA) and Defra introduced the Zoonoses Action Plan (ZAP) *Salmonella* monitoring programme which was in operation until April 2008. Under this scheme, the *Salmonella* sero-prevalence in finisher pig herds was measured using the *Salmonella* meat-juice ELISA test (Hill *et al*., 2008). Based on the antibody levels, farms were assigned to 3 categories and control measures were required at herds with highest sero-positivity (level 2 and 3). In November 2003, Regulation (EC) No 2160/2003 on the control of *Salmonella* and other specified food-borne agents came into force (Anonymous, 2003) in order to protect; the principle of the regulation is for the whole food chain to be controlled (Anonymous, 2003). As the requirement for all EU Member States to have a National Control Plan for *Salmonella* in pigs ZAP *Salmonella* scheme was replaced by a wider scheme – Zoonoses National Control Program for *Salmonella* in pig meat (ZNCPig) in April 2008. The ZNCPig takes action at every level in the chain from farm to fork in order to reduce the risk from *Salmonella* in pork and pork products (Belsue *et al*., 2011; Clough *et al*., 2009).

1.4 Controlling *Salmonella* – multiple strategies

1.4.1 Biosecurity and disinfection

Salmonella infections in animals are controlled by a number of means. Appropriate grouping of animals and applying all-in, all-out production systems can minimize spread of *Salmonella* infection (Beloeil *et al*., 2007). Reducing carriers such as, rodents, birds, vehicles and visitors decreases the likelihood of cross-contamination between houses or farms (Lo Fo Wong *et al*., 2002). The reduced sero-prevalence of *Salmonella* was associated when holdings were equipped with staff showers and changing room facilities (Lo Fo Wong *et al*., 2004). Importance of effective cleaning and disinfection has been reported in various publications, however studies have also shown that disinfectant exposure can create a selective pressure and decreased susceptibility to antibiotic (Randall *et al*., 2007; Randall *et al*., 2004). The same authors observed that the disinfectant-selected mutant could colonise the chicken equally to the control strain, thus persisting in the host and environment (Randall *et al*., 2007).

1.4.2 Antimicrobials

Intensive farm production, overfeeding, overpopulation and stress can predispose to acute or chronic diseases and to changes in microbiological status of the gut flora. In order to try and address the consequences of intensification antimicrobial growth promoters were introduced in the 1940's (Page, 2006). Beneficial effects of antimicrobial growth promoters such as better weight-gain ratio and more uniform growth resulted in their frequent use in the farming industry (Delsol *et al*., 2004; Page, 2006). Consequently, prolonged use of antimicrobials in veterinary medicine and animal husbandry has resulted in the adaptation of pathogenic bacteria evolving resistance mechanisms (Boerlin and Reid-Smith, 2008). Antimicrobial resistance (AMR) can be acquired via horizontal gene transfer or mutations of genes already present in bacteria. Several mechanisms are involved in the horizontal gene transfer (HGT). Intracellular gene movement is via transposons which can move within the genome and integrons. Integrons can encode mechanisms to gain and/or excise genes, thus moving resistant gene cassette. Frequent transmission of resistance genes from one bacterium to another is by plasmids and bacteriophages (Boerlin and Reid-Smith, 2008). The importance of genomic islands (SGI1) in the spread of penta-resistant *S.*

Typhimuirum has been widely reported (Threlfall, 2000). The typical pattern of penta-resistance is characterised by resistance to ampicillin, chloramphenicol, streptomycin, sulphonamide and tetracycline (ACSSuT). A number of reports have demonstrated acquired resistance to more antimicrobials in DT104 e.g. fluoroquinolones (Threlfall, 2000). The isolation of *S.* Typhimurium DT104 in the UK from humans dates back to 1980 (Threlfall *et al*., 1994) and currently constitutes an increasing public health problem. Concerns about increased resistance and multidrug resistance in bacteria and particularly those that are zoonotic resulted in the withdrawal of antimicrobial growth promoters in an animal feeds in EU in 2006, thus the need of alternative in feed control measures is evident.

1.4.3 Feed incorporated control strategies

Growing concern regarding antimicrobial resistance in bacterial pathogens led first to restrictions and then a total ban of antibiotics as growth promoters in animal feed in EU. Subsequently, a need for utilization of a various alternative intervention strategies increased. One such alternative is use of probiotics, live microbial supplements, which can be administered as defined mono-strain, multi-strain cultures, multispecies or undefined mixed cultures (Bomba *et al*., 1998; Callaway *et al*., 2008b; La Ragione *et al*., 2001; Stavric and d'Aoust, 1993; Timmerman *et al*., 2004). Probiotics can be administered to the animal in the pelleted feed or as powder, granules or encapsulated (Fuller, 1989). Microorganisms used are those belonging to *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Enterococcus*, *Pediococcus*, *Streptococcus* genera, but also avirulent *E. coli* and yeasts belonging to the *Saccharomyces cerevisiae* species (Anadon *et al*., 2006; Vondruskova *et al*., 2010) and currently there are several commercially available veterinary products which have been considered as safe to use in the EU (SCAN, 2003). There are multiple possible mechanisms by which probiotics confer health benefits to the host. Those include competition for nutrients and binding sites, production of a variety of antimicrobial compounds, alteration of microbial metabolism and immunity stimulation (Delcenserie *et al*., 2008; Fuller, 1989; Rowland *et al*., 2010), nevertheless the full array of mechanisms has not yet been elucidated. The subject of probiotic use in livestock with regards to their ability to control infections is continuously investigated. Numerous studies have demonstrated that various probiotic strains reduce foodborne pathogens in pigs and poultry (Casey *et al*., 2007; Higgins *et al*., 2010; Konstantinov *et al*., 2008;

Nemcova *et al*., 2007; Stern *et al*., 2001). Despite, however, many scientific reports of probiotic efficacy against pathogens and health benefits, some authors have not seen such effects (Kreuzer *et al*., 2012; Szabo *et al*., 2009).

In addition to probiotics, a range of prebiotics are also administered alone to enhance certain bacterial populations or in combination with the selected probiotic (Macfarlane *et al*., 2008; Patterson and Burkholder, 2003). Other effects of prebiotics might include host immunomodulation, inhibition of intestinal colonisation by pathogens, also improve feed intake and animal weight gain (Collins *et al*., 2009) and will be discussed in the subsequent sections.

Organic acids are regularly included in animal diets as a preservative and alternative control strategy for pathogen reduction. It is reported that addition of organic acids lowers pH, increases proteolysis and nutrient digestibility, also reduces numbers of pathogenic bacteria and as a consequence improves animal health, diet utilization and growth in pigs and poultry (Mroz, 2005; Van Immerseel *et al*., 2006). In pigs both antimicrobial activity and growth performance improvement was reported with the use of organic acids, especially during weaning and post-weaning period (Partanen *et al*., 2006; Tsiloyiannis *et al*., 2001). The efficacy of this treatment differs and is dependent on various factors, such as type of acid, concentration and pH and encapsulation (Boyen *et al*., 2008b; Foegeding and Busta, 1991; Papatsiros *et al*., 2012).

1.4.4 Bacteriophages

Another prospective intervention strategy to reduce pathogen carriage is thought to be application of lytic bacteriophages, specific for foodborne pathogens (Doyle and Erickson, 2012). Bacteriophages recognise specific bacterial outer membrane receptors, then infect their DNA into a host bacterium, replicate within and then cause bacterial cell lyses only releasing newly formed virions (Doyle and Erickson, 2006). Several studies have confirmed their efficacy against *E. coli* and *Salmonella* spp. in various animal species (Raya *et al*., 2011; Smith and Huggins, 1983; Wall *et al*., 2010) and others report that therapeutic effect is dependent on the route of administration (Huff *et al*., 2002a; Huff *et al*., 2002b). The specificity of bacteriophage and their ability to self-replicate only in the target host bacterium is an attractive potential control mechanism (Sulakvelidze *et al*., 2001). However, further research is needed as several concerns regarding to their efficacy have been raised including development of

phage-neutralizing antibodies, phage resistance or possibility of bacterial toxin genes transfer by bacteriophages (Matsuzaki *et al*., 2005; Sulakvelidze *et al*., 2001).

1.4.5 Vaccination

The aim of the vaccination is to mimic the development of immunity by inoculation of avirulent but still immunogenic whole cells or components of the pathogen (Meeusen *et al*., 2007). The vaccine should in consequence prevent colonisation, reduce shedding by infected pigs and increase threshold for infection of susceptible pigs with *S*. Typhimurium (Haesebrouck *et al*., 2004).

Live attenuated vaccines seem to give better protection due to the stimulation of the cellular and humoral immune systems. Of the live vaccines a number have been gene knock-outs such as *aroA, cya* and *crp,* these vaccines have been tested for use as live vaccines in mice, poultry and pigs (Barrow *et al*., 2001; Barrow and Wallis, 2000; Curtiss and Kelly, 1987). A metabolic drift mutant of *S*. Typhimurium (*gyrA-cpxA-rpoB*) has proven to be successful in reducing colonisation by wild type *Salmonella* strains in poultry (Linde *et al*., 1996). The same mutant-based vaccine was used in young piglets, followed by challenge with *S.* Typhimurium DT104 strain and as previously, a significant reduction in colonisation and shedding was observed (Roesler *et al*., 2004). In the further study, a reduction of *Salmonella* shedding in piglets after inactivated herd-specific *Salmonella* vaccine was used in pregnant sows was demonstrated (Roesler *et al*., 2006). However, an attenuated *aroA S*. Typhimurium mutant failed to produce a protective response in gnotobiotic pigs against *S*. Typhimurium LT2 challenge (Trebichavsky *et al*., 2006).

Efficient vaccination could be useful to control *Salmonella* on farm, however the efficacy in reducing prevalence is not yet fully demonstrated (Denagamage *et al*., 2007). Moreover, the inability to differentiate between vaccinated and infected animals could be a problem, as the interpretation of serological test results might occur and confound statutory testing. A negative-marker vaccine against *Salmonella* that enables the separation of infected from vaccinated animals has been effective in reducing clinical signs and colonisation in pigs challenged with *S*. Typhimurium (Selke *et al*., 2007).

1.5 The gut microbiota - probiotics, prebiotics and synbiotics

1.5.1 Importance of gastrointestinal microbiota

The gastrointestinal tract (GIT) is a complex ecosystem forming a border between the outside world and extracellular host fluids; it is also the main gate of entry for enteropathogens.

Humans and animals are born without gastrointestinal microflora, however, colonisation by a microbiota derived from the mother and the environment begins immediately after birth (Conway, 1997; Servin, 2004). The order in which colonisation begins in human starts with aerobic and facultative bacteria e.g. coliforms and then streptococci followed by the later appearance of bifidobacteria, lactobacilli and clostridia spp. (Dibner *et al*., 2008; Mackie *et al*., 1999). Similarly, piglets become rapidly colonised, firstly with aerobes and facultative anaerobes and the numbers reach $10^9 – 10^{10}$ bacteria/g of colonic content after 12 hours after birth (Swords *et al*., 1993). Those very early aerobic and facultative anaerobes colonisers constitute 80% of the total microflora, however as soon as 48 hours after birth facultative anaerobes are displaced by obligate anaerobes, which then account for 90% of the microflora (Swords *et al*., 1993). From then on, this gastrointestinal ecosystem of suckling piglets remains established until the weaning period. Weaning is a very stressful period and results in significant changes in the intestinal microflora with most noticeably decreases in the number of lactobacilli (Pieper *et al*., 2006; Pieper *et al*., 2008).

Establishment of a gastrointestinal microflora plays an important role in development of intestinal functions, including maturation of the immune system (Isolauri, 2001). It has been reported that the host-microbial relationship is mutual and that gut-associated lymphoid tissue (GALT) can affect the gut bacterial composition (Xu and Gordon, 2003). The presence of the balanced GIT microflora facilitates its normal function in both human and animal health (Rakoff-Nahoum *et al*., 2004; Servin, 2004; Xu and Gordon, 2003). The large intestine is occupied by various groups of bacteria, which can both have positive and negative effects on the host health (Figure 1.1). During homeostasis, both of these groups are involved in fermentation of non-digestible for the host products (Gibson and Roberfroid, 1995). Interestingly, in pig, LAB bacteria are associated with the non-secreting area of the stomach (*pars oesophega*), of which cells are continuously desquamating (Fuller *et al*., 1978). As a results LAB are being continuously released into the lumen, which as has been

suggested could contribute to control of enteropathogenic bacteria (Fuller, 1992).

It has been suggested that early exposure to environmental bacteria is able to affect the presence of certain groups of bacteria in the GIT, thus colonisation of the host with fully developed bacterial GIT population may be less effective (Mackie *et al*., 1999). The need of host early exposure to numerous microorganisms, especially the health-promoting ones, is important as mentioned already for development of immune system and intestine architecture (Round and Mazmanian, 2009). Antibiotics alter the gut flora and when used therapeutically this should be taken into account and recovery strategies developed. Suitable, accurately designed feeding strategies including probiotics, prebiotics and synbiotics could therefore facilitate the normal physiological development of an animal but also guard the gut homeostasis thereafter and contribute to disease prevention.

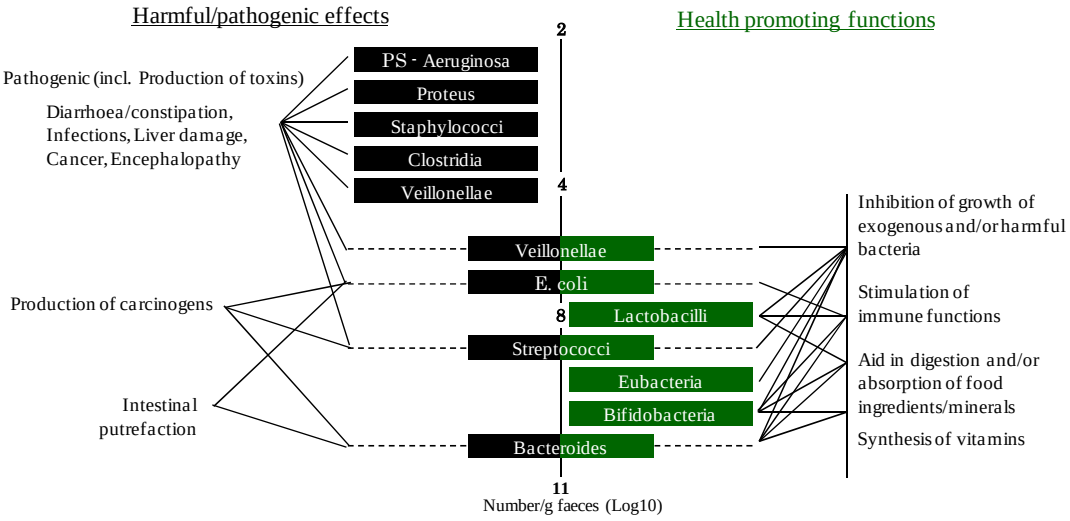


Figure 1.1 The composition and health effects of predominant human faecal bacteria and their possible effect on the host. Adapted from Gibson and Roberfroid (1995).

1.5.2 Defining probiotics, prebiotics and synbiotics

The term ‘probiotic’ was first used in 1965 by Lilly and Stillwell (Lilly and Stillwell, 1965; Schrezenmeir and de Vrese, 2001) to describe substances produced by various protozoan species that stimulated growth of other protozoan species. By 1974, Parker defined probiotics as “organisms and substances which contribute to intestinal microbial balance” (Fuller, 1989; Parker, 1974). This definition was further modified by Fuller (1989) due to its imprecise form and to highlight the importance of live cells.

Thereafter the definition was read as "live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". As scientific knowledge about probiotics widened, the definition was also expanded by others (Schrezenmeir and de Vrese, 2001), nevertheless the Fuller version has been most widely used. Recently, it was again updated to “a preparation of viable microorganisms, which is consumed by humans or other animals with the aim of inducing beneficial effects by qualitatively or quantitatively influencing their gut microbiota and/or modifying their immune status” (Cartman *et al*., 2008; Fuller, 2004).

In 1995, Gibson and Roberfroid introduced the concept of prebiotics and termed them as “non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/ or activity of one or a limited number of bacteria in the colon” (Gibson and Roberfroid, 1995).

The same authors encouraged further research and definition of a combination of pro and prebiotics, namely synbiotics, as “a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare” (Gibson and Roberfroid, 1995).

1.5.3 Probiotics

1.5.3.1 History of probiotic development and use in humans and livestock

The concept of probiotics as we understand it today was first introduced by a Russian scientist Élie Metchnikoff, who suggested that enhanced health and longevity of Bulgarian peasants was attributed to the consumption of fermented dairy products containing lactobacilli (Metchnikoff, 1907). This however, was not the first reference claiming health benefits due to consumption of live microorganisms in the food and most probably the oldest dates back to a Persian version of the Old Testament (Genesis 18:8) (Bottazzi, 1983; Schrezenmeir and de Vrese, 2001). Those health benefits were not only referred to lactobacilli. Henry Tissier, a French pediatrician isolated *Bifidobacterium* species from the gut of breast-fed infants and suggested that those when administered to infants suffering from diarrhoea could displace putrefactive bacteria and restore healthy microflora (Tissier, 1906). Furthermore, German Professor Alfred Nissle isolated and demonstrated the use non lactic acid bacterium *E. coli*

(Nissle, 1916). To date *E. coli* Nissle 1917 is commonly used as a probiotic strain (Altenhoefer *et al*., 2004; Schierack *et al*., 2011). Later on, in 1935 *Lactobacillus acidophilus* was proposed by Rettger to be an appropriate species to treat human disorders (Rettger *et al*., 1935).

Since those early days the concept of probiotics flourished, and in humans probiotics are often been administered as a functional food showing a variety of beneficial effects (Andersson *et al*., 2001; Kotowska *et al*., 2005; Ruszczyński M, 2008). Moreover, the concept of alternative strategies to control gastrointestinal pathogens in livestock such as *S*. Typhimurium is a popular area of research. In order to replace antimicrobials, probiotic, prebiotics, competitive exclusion cultures (CE) and organic acids (Callaway *et al*., 2008a; Cho *et al*., 2011; Papatsiros *et al*., 2012) are accepted as such alternatives. In pigs, probiotic bacteria have not only been used to control microbial balance in disease control, but also as growth-promoters, improving efficiency of digestion (Cho *et al*., 2011; Collins *et al*., 2009).

1.5.3.2 Design and selection of probiotics

With the continuing scientific interest and commercial use of probiotics both in humans and animals the precise assessment with regards to a specific selection criteria and safety is needed. It is often a part of scientific research providing the information about the uniqueness of a particular probiotic isolate. According to Klaenhammer and Kullen (1999) all criteria for the probiotic selection can be functionally collated into four categories, namely appropriateness, technological suitability, competitiveness, performance and functionality. Those criteria are the outcome of over 20 years of recommendations and many comprehensive scientific publications are available.

A trustworthy probiotic requires thorough and correct identification using current molecular methodology, should have generally regarded as safe (GRAS) status and be of host origin (Gueimonde and Salminen, 2006; Klaenhammer and Kullen, 1999). Within the European Union (EU) safety of probiotics as animal feed additives is regulated by Regulation 18131/2003 EU and in accordance with guidelines of scientific committees (von Wright, 2005). Currently, European Food Safety Authority (EFSA), which overtook the functions of the scientific committees in the food and feed areas, is providing independent scientific advice to the European Commission (EC) (Anadon *et al*., 2006; von Wright, 2005). Previously, for animal feed additives the advice and guidelines were of responsibility of Scientific Committee of Animal

Nutrition (SCAN). Probiotic safety has to be assessed according to the guidelines defined in Council Directive 87/153/EEC (von Wright, 2005). The directive states that the probiotics must not produce toxins, virulence factors, antibiotic substances of clinical importance or carry transmissible antibiotic resistance determinants. The expression and transferability of antimicrobial resistance determinants from probiotics to the commensal bacteria present in the gut is an important concern (Sanders *et al*., 2010), and the assessment of antibiotic sensitivities and deduction of the genetic basis of any resistance prior to its clinical use are vital components of the safety assessment. Some genes responsible for the resistance of specific antimicrobials are identical among pathogenic and commensal LAB (Teuber *et al*., 1999). In general LAB antibiotic resistance is of intrinsic nature although infrequent plasmid-linked resistances are observed (Salminen *et al*., 1998). The technical guidance prepared by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), which updated previously adopted SCAN opinion "on the criteria for assessing the safety of micro-organisms resistant to antibiotics of human clinical and veterinary importance" sets the minimum inhibitory concentrations (MICs) for the commonly used antimicrobials (EFSA, 2008b).

The ability of a probiotic to withstand various *in vivo* stresses, including acid, bile, and antimicrobial compounds produced by other gut bacteria plus the ability to adhere to and colonise gut epithelium are all part of the competitiveness selection criteria. Performance and functionality criteria concentrate mainly on probiotic antagonistic effects towards pathogens, production of bioactive compounds and immunodulation. Numerous scientific reports focusing on evaluating those criteria have been published to date (Bielecka *et al*., 2002; Casey *et al*., 2007; Dunne *et al*., 2001; Gueimonde and Salminen, 2006; Hyronimus *et al*., 2000; Ljungh and Wadstrom, 2006). Nevertheless, as the mechanisms of the functional roles of probiotics remain unclear, the importance of the competitiveness and performance criteria mentioned above are questioned by others (Klaenhammer and Kullen, 1999).

1.5.3.3 Mechanism of probiotic action

The mechanism of probiotic action has not been fully elucidated. However, it is clear that multiple physiological, antimicrobial and immunomodulatory mechanisms are involved (Gill, 2003; Servin, 2004). Competition for nutrients, restriction of and competition for receptor sites, immunomodulation and production of antimicrobial

substances are all possible mechanisms by which effectiveness of CE cultures can be explained (Doyle, 2001; Mead, 2000). Furthermore, the effect of the gut microbial community as a whole on the host health is very important. The mammalian–microbial symbiosis could affect the metabolism of endogenous and exogenous compounds and be important in disease etiology (Dumas *et al*., 2006; Martin *et al*., 2007). In fact, *in vivo* all those factors synergistically contribute to the effectiveness of probiotics.

The concept of "competitive exclusion" was introduced by Nurmi and Rantala (1973) following observations that administration of intestinal contents from adult birds to a chicks resulted of their increased resistance to *Salmonella* infection. Interestingly, over 20 years prior to introduction of the CE term, the age related reduction in susceptibility to experimental *Salmonella* was well known (Milner and Shaffer, 1952). The form of CE is mucosal competitive exclusion (MCE), which follows the idea that deeper tissue scraping could be more efficient against pathogen colonisation and was first developed in order to control *Campylobacter* colonisation (Stern *et al*., 2001). The same authors reported that in birds challenged with *S*. Typhimurium CE cultures were less protective than used MSC cultures (Stern *et al*., 2001). Moreover, Fedorka-Cray *et al*. (1999) developed a mucosal competitive exclusion culture from swine (MCES) which was linked with reduction of *S*. Choleraesuis in caecal contents and ileocolic junction compared with the un-treated group. The prevention or reduction of pathogens by CE has been acknowledged in the scientific community and studied continuously, particularly with regards to pathogen control in poultry (Fedorka-Cray *et al*., 1999; Genovese *et al*., 2000; La Ragione *et al*., 2001; La Ragione *et al*., 2004; La Ragione and Woodward, 2003; Zhang *et al*., 2007a, b). In chicks it has been shown that CE treatment could be effective against pathogens such as pathogenic *E. coli*, *C. jejuni*, *Listeria monocytogenes* and *C. perfringens* (Hakkinen and Schneitz, 1996, 1999; Hume *et al*., 1998; La Ragione *et al*., 2004; Nisbet, 2002; Schoeni and Wong, 1994). To date both undefined and defined CE cultures have been used to evaluate efficacy against *Salmonella* in poultry and swine and several commercial products are available (Schneitz, 2005; Stavric and d'Aoust, 1993).

Production of a wide range of antimicrobial compounds such as organic acids, mainly lactate and acetate; inhibitory peptides and hydrogen peroxide has been reported as an effective means against many pathogenic bacteria, fungi and viruses. Carbohydrates fermentation by lactobacilli results in accumulation of fermentation

products, mainly lactic, acetic acid but also ethanol, and formic acid, dependant on the type of fermentation (Collins *et al*., 2009). The inhibition of growth of bacterial pathogens in the presence of organic acids such as lactate or acetate has been demonstrated (Adams and Hall, 1988; Makras *et al*., 2006; Servin, 2004). The antibacterial mechanism of lactic acid has not only been attributed to lowering the pH and diffusion of the un-dissociated molecule across the bacterial cell membrane, but also it has been reported that lactate acts as an outer membrane permeabilizer enabling effective penetration of the numerous other than organic acids antimicrobial metabolites produced by the lactobacilli (Alakomi *et al*., 2000; De Keersmaecker *et al*., 2006; Eklund, 1983). Studies of Makras *et al*. (2006) reported antibacterial activity of *L. johnsonii* La1 and *L. plantarum* ACA-DC 287 against *S.* Typhimurium that was due to unknown compounds that were effective in the presence of lactic acid. Coconnier-Polter *et al*. (2005) reported *S.* Typhimurium SL1344 sensitization to lytic compounds following exposure of to the cell free supernatant of *L. acidophilus*. It was thereafter concluded that this antibacterial activity was due to the non-lactic-acid molecule. In the dynamic environment of the gastrointestinal tract, the antagonistic activity of probiotics is far more complex, due to modulation of both organic acids concentration and members of microbial community. Moreover, the presence of other bacterial genera would result in the presence of other than lactic and acetic acids, such as butyrate, propionate, formate (Belenguer *et al*., 2006; Macfarlane and Macfarlane, 2007; Ushida *et al*., 2002) and those can modulate bacterial pathogenicity (Boyen *et al*., 2008b; Gantois *et al*., 2006; Van Immerseel *et al*., 2006).

In addition to organic acids, lactic acid bacteria can produce several antimicrobial metabolites that can be divided based on their molecular mass into bacteriocins and low molecular mass compounds (Niku-Paavola *et al*., 1999).

Bacteriocins are a heterogeneous group of peptides and proteins with regards to their size and function and their classification is continuously revised with several different bacteriocin classes and subclasses (Beshkova and Frengova, 2012; Cotter *et al*., 2005). Bacteriocins produced by LAB show promise as candidates for pathogen control (Cotter *et al*., 2005). However, the activity of these heat-stable peptides is mainly directed against Gram-positive bacteria, unless the integrity of the outer membrane Gram-negative species has been compromised (Ouwehand and Vesterlund, 2004).

Low molecular antimicrobial substances produced by some *L. reuteri* strains in

the presence of glycerol (Axelsson *et al*., 1989) has attracted intensive interest. Reuterin that is water-soluble, active at a wide pH range, resistant to proteolytic and lipolytic enzymes (Rodriguez *et al*., 2003), has been shown to have activity against Gram-negative and Gram-positive bacteria and also against yeast, fungi or protozoa (Axelsson *et al*., 1989; Spinler *et al*., 2008). The presence of reuterin producing *L. reuteri* in proximal regions of the swine gastrointestinal tract has been shown (Axelsson *et al*., 1989). Another low molecular weight, antimicrobial compound produced by some *L. reuteri* strains is reutericyclin, a tetramic acid derivative which is structurally related to tenuazonic acid (Ganzle *et al*., 2000). It is bacteriostatic or bactericidal to Gram-positive bacteria, whereas Gram-negative bacteria are resistant to its activity (Ganzle, 2004).

The ability of certain *Lactobacillus* species to produce hydrogen peroxide with a sufficient oxygen supply has been long-established (Antonio *et al*., 2005; Eschenbach *et al*., 1989; Song *et al*., 1999). Numerous studies showed antimicrobial activity of hydrogen peroxide producing lactobacilli on various pathogens (Dahiya and Speck, 1968; Klebanoff *et al*., 1991). The bactericidal effect of hydrogen peroxide has been ascribed to its strong oxidizing effect on the bacterial cell (Ouwehand and Vesterlund, 2004). Pridmore *et al*. (2008) demonstrated *in vitro* killing activity of the hydrogen peroxide produced by *L. johnsonii* strain NCC 553 towards *S.* Typhimurium SL1344. However, the presence of hydrogen peroxide producing lactobacilli has been linked with the displacement of pathogens and the maintenance of homeostasis of the normal vaginal flora (Eschenbach *et al*., 1989; Felten *et al*., 1999; Martin *et al*., 2008). In the human gut, the presence hydrogen peroxide of bacterial origin has been linked with apoptosis of colonocytes (Strus *et al*., 2009).

Dysbiosis of the gut microflora can increase antigen transport across the gut mucosa (Isolauri *et al*., 2001). It is postulated that probiotics can enhance the mucosal epithelial cell barrier via release of bioactive compounds or indirectly through immune cells activation, thus improving host defenses and preventing pathogens from attaching to the epithelium (Deplancke and Gaskins, 2001; Saulnier *et al*., 2009a). Probiotics are recognised by toll-like receptors (TLRs) in the gut epithelial cells and/or antigen-presenting cells and they stimulate a cascade of immunological events including production of cytokines by enterocytes such as IL-8, IL-6 (Delcenserie *et al*., 2008; Rakoff-Nahoum *et al*., 2004; Walker, 2008). The microbiota present in the gut also exerts effect through balanced control of both pro and anti-inflammatory responses,

however, the exact mechanism and the contribution of probiotics to immune modulation is not yet fully understood (Fleige *et al*., 2009; Isolauri *et al*., 2002; Round and Mazmanian, 2009). The fact that probiotic immune modulation is very complex with each specific strain altering the specific immune responses, highlights the need for candidate probiotics to be described accurately as suggested by Delcenserie *et al*. (2008) with regards to the cytokine profiles secreted by lymphocytes, enterocytes and/or dendritic cells.

1.5.4 Prebiotics

1.5.4.1 Prebiotics concept in human and livestock

Bifidobacteria can utilize complex oligosaccharides, resulting in decreased faecal pH and increased bifidobacteria numbers (Mitsuoka *et al*., 1987; Yazawa *et al*., 1978). In 1995 Gibson and Roberfroid defined prebiotics as "non-digestible food ingredients that beneficially affects the host by selectively stimulating the growth and/ or activity of one or a limited number of bacteria in the colon" (Gibson and Roberfroid, 1995). As this definition regarded only human colonic environment it was updated in 2004 by Gibson *et al*. (2004). Restructured definition stated that "A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host wellbeing and health". Numerous "colonic foods" can be used as a substrate for endogenous bacteria, yet they cannot be classified as prebiotics (Gibson and Roberfroid, 1995). To be defined as prebiotic, each food ingredient must demonstrate resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption. Moreover, it must be fermented by the intestinal microflora with selective stimulation of the growth/or activity of intestinal bacteria that are associated with health and wellbeing (Gibson *et al*., 2004). To asses if a prebiotic fulfills these criteria, especially its resistance to low pH, hydrolysis and absorption both *in vitro* and *in vivo* methodology are applied (Gibson *et al*., 2004). Moreover, to date several *in vitro* systems have been applied to evaluate candidate prebiotic fermentation and selective stimulation properties. Those include simple pure mono or mixed culture studies, batch culture systems inoculated with the faecal slurry and continuous culture systems, mimicking various parts of colon (Macfarlane and Macfarlane, 2007; Macfarlane *et al*., 1998; Rycroft *et al*., 2001). Regardless of complexity of an *in vitro* method employed, a

feeding trial should be included as a concluding demonstration of prebiotic efficacy (Roberfroid, 2008). Fermentation of prebiotics by intestinal microbiota is linked with increase in the numbers of selectively enhanced bacterial groups, mainly lactobacilli and bifidobacteria (Collins and Gibson, 1999).

Prebiotics are considered as safe, as they can be found in existing foods range such as bananas, onions, artichokes and human breast milk, nevertheless considerations should be taken into account when the maximum dose is administered (Tuohy *et al*., 2003). The most commonly studied nondigestable carbohydrates in both humans and animals are inulin, fructooligosaccharides (FOS) (Nemcova *et al*., 2007; Roberfroid, 2000; Sabater-Molina *et al*., 2009; Tsukahara *et al*., 2003), galactooligosaccharides (GOS) (Searle *et al*., 2009; Searle *et al*., 2010; Vulevic *et al*., 2008) and lactulose (Bouhnik *et al*., 2004; Fleige *et al*., 2007, 2009; Martin-Pelaez *et al*., 2010). Nevertheless, other such as lactitol, raffinose and mannanoligosaccharides (MOS) (Castillo *et al*., 2008; Zentek *et al*., 2002) have also been evaluated. Commercially available prebiotics of confirmed efficacy are FOSs and inulin, GOSs and lactulose (Tuohy *et al*., 2003). Interestingly, although in humans lactulose is conventionally used as a laxative to treat constipation, in small doses has proven to have beneficial effects on the human colonic bifidobacteria (Tuohy *et al*., 2005). In animals several studies aiming to evaluate the effect of prebiotic administration demonstrate their beneficial effects associated with modulation of gut bacterial microbiota and the enhancement of the increase in the SCFA concentrations (Smiricky-Tjardes *et al*., 2003; Yusrizal and Chen, 2003), reduction of intestinal colonisation by pathogens (Bovee-Oudenhoven and Van der Meer, 1997; Collins *et al*., 2009; Lowry *et al*., 2005; Searle *et al*., 2009) and increase in animal performance (Pierce *et al*., 2005; Sims *et al*., 2004; Verdonk *et al*., 2005).

1.5.4.2 Prebiotics fermentation by gut microbiota

Due to the greater availability of carbohydrate in the proximal colon, this is the active site of carbohydrate fermentation whereas the subsequent parts of the large intestine are proteolytic as the substrate availability diminishes (Macfarlane *et al*., 1992). Fermentation of accessible carbohydrates in the colon results in production of short chain fatty acids (SCFA), lactate and various other end metabolites such as ethanol, succinate, hydrogen, carbon dioxide and hydrogen sulfide (Cummings, 1981; Gibson *et al*., 1996).

SCFA, mainly acetate, propionate, butyrate are the major end products of microbial fermentation and are fundamental to many biological functions, not only have a positive effect on digestion, but also on host immunity and metabolism (Gibson, 1999; Macfarlane and Macfarlane, 2007; Saulnier *et al*., 2009b). The uptake of the SCFA in the colon seems to be in a concentration-dependent manner and is associated with increased water, calcium and magnesium absorption (Topping and Clifton, 2001; Yanahira *et al*., 1997). Following absorption, SCFAs are metabolised in the cecocolonic epithelium, muscle and liver cells (Gibson, 1999; Wong and Jenkins, 2007). The role of the active end-products of bacterial fermentation is illustrated in the Table 1.2. In particular, butyrate was found to be very important as it acts as an energy source, signaling metabolite, proliferation stimulus for colonic epithelial cells and an anti-proliferative signal for neoplastic colonocytes (Topping and Clifton, 2001). Interestingly, in a complex microbial environment production of butyrate is not only determined by the ability of particular bacteria to utilise the prebiotic directly, but also indirectly through a cross-feeding on metabolic products such as lactic acid by other bacteria (Belenguer *et al*., 2006).

Table 1.2 Gut bacteria and their metabolic products of carbohydrate fermentation. Adapted from Gibson (1999).

End product	Bacterial group involved	Metabolic designation
Acetate	Bacteroides, bifidobacteria, eubacteria, lactobacilli, clostridia, ruminococci, peptococci, veillonella, peptostreptococci, propionibacteria, fusobacteria	Metabolised in muscle, kidney, heart and brain
Propionate	Bacteroides, propionibacteria, veillonella	Cleared by the liver, possible gluceogenic precursor, suppresses cholesterol synthesis
Butyrate	Clostridia, fusobacteria, butyrivibrio, eubacteria, peptostreptococci	Metabolised by the colonic epithelium, regulator of cell growth and differentiation
Ethanol, succinate, lactate, pyruvate	Bacteroides, bifidobacteria, lactobacilli, eubacteria, peptostreptococci, clostridia, ruminococci, actinomycetes, enterococci, fusobacteria	Absorbed, electron sink products, further fermented to short-chain fatty acids
Hydrogen	Clostridia, ruminococci, fusobacteria	Partially excreted in breath, metabolised by hydrogenotrophic bacteria

1.5.4.3 Therapeutic effects of prebiotics

There are numbers of proposed mechanisms by which prebiotics exert their therapeutic effect, such as selective stimulation of favorable bacterial species,

increased production of organic acids, direct pathogen reduction and stimulation of the host immune system (Collins *et al*., 2009; Macfarlane *et al*., 2008). Growth stimulation of intestinal microbiota, increased concentrations of organic acids and reduced pH in the gut has been linked with inhibitory properties on pathogenic bacteria (Collins and Gibson, 1999; Sako *et al*., 1999; Skrivanova and Marounek, 2007). It has been reported that butyric and propionic acid can decrease intestinal colonisation by *S.* Enteritidis in poultry (Van Immerseel *et al*., 2005; Van Immerseel *et al*., 2006). Similarly, recent studies have shown that even low concentrations of especially butyric and propionic acids decreased virulence gene expression of *S.* Typhimurium and decreased faecal shedding and intestinal colonisation of pigs (Boyen *et al*., 2008b). Some prebiotics can directly prevent pathogen adherence as they resemble or mimic host oligosaccharide receptors (Shoaf-Sweeney and Hutkins, 2008), thus preventing colonisation and infection. Prebiotics that act as receptor analogues for a pathogen include human milk oligosaccharides (HMOs), galactooligosacharides (GOSs) and mannanoligosaccharides (MOS) (Castillo *et al*., 2008; Kunz *et al*., 2000; Quintero *et al*., 2011; Tzortzis *et al*., 2005). It has been demonstrated that GOS formulations have the potential to reduce the adherence of enteropathogenic *E. coli* and *S.* Typhimurium *in vitro* and *in vivo* (Searle *et al*., 2009; Searle *et al*., 2010; Tzortzis *et al*., 2005).

In addition, it has been suggested that prebiotics may affect the immune system either directly or indirectly, through enhanced growth of specific intestinal bacterial groups (Roberfroid *et al*., 2010; Vos *et al*., 2007). Increased caecal secretion of IgA was reported in rats fed glucomannan and lactulose (Kudoh *et al*., 1999). In the study of Agunos *et al*. (2007) increased *S.* Enteritidis specific secretory IgA (sIgA) concentrations were observed in caecal and bile contents of chicks fed a diet supplemented with β1–4 mannobiose or D-mannose, which was linked with a decline in shedding and caecal carriage of the pathogen. Various human intervention and animals studies indicate prebiotics ability to modulate immune system (Fleige *et al*., 2009; Janardhana *et al*., 2009; Macfarlane *et al*., 2008; Nagura *et al*., 2002). The exact mechanism of their action has not yet been fully elucidated, nevertheless few means have been suggested and summarised by Seifert and Watzl (2008). Those include interaction of prebiotics with carbohydrate receptors on leukocytes or their partial absorption resulting in local and systemic contact with the immune system. Furthermore, alteration in particulate bacterial group and in turn modulation in production of cytokines and antibodies, increased SCFA production and their

enhanced binding to G-protein on leucocytes has also been proposed as potential mechanisms (Seifert and Watzl, 2008).

1.5.5 The use of probiotics, prebiotics and synbiotics in pigs

It has been demonstrated that the administration of probiotics (Fuller, 1989; Ohashi *et al*., 2007; Scharek *et al*., 2007b; Takahashi *et al*., 2007; Vondruskova *et al*., 2010) and prebiotics (Bauer *et al*., 2001; Houdijk *et al*., 2002; Konstantinov *et al*., 2004; Martin-Pelaez *et al*., 2010; Smiricky-Tjardes *et al*., 2003; Tzortzis *et al*., 2005) have stimulatory effect on the gut microbiota of pigs. Beneficial effects of probiotics (Lessard *et al*., 2009; Scharek *et al*., 2007a; Schierack *et al*., 2007) and prebiotics (Jung *et al*., 2004; Pie *et al*., 2007) related to their immunomodulatory properties have also been reported. Moreover, it has been showed that the administration of probiotics and prebiotics in pigs can improve digestion, pig growth performance and feed efficiency (Davis *et al*., 2004; Estrada *et al*., 2001; Huang *et al*., 2004; Nousiainen *et al*., 2004). Inclusion of LAB complexes comprising of *E. faecium*, *P. pentosaceus*, *L. plantarum* and *L. acidophilus* resulted in significantly increased daily feed intake, weight gain and feed conversion ratio during the first two weeks after weaning (Giang *et al*., 2010). The concept of synbiotics is particularly interesting, as prebiotics confer beneficial effects to the administered probiotic, other resident microflora and the host. One such study reported that in weanling pigs, the effect of the administration of *L. paracasei* together with oligofructose was better than using the probiotic alone and resulted in a reduction in clostridia and enterobacteria but increased the beneficial lactobacilli and bifidobacteria (Nemcova *et al*., 1999). Similarly, Bomba *et al.* (2002) found that superior to *L. paracasei* alone, the use of *L. paracasei* and FOS synbiotic combination had beneficial effects on bacterial population of weanling pigs, and particularly lactobacilli and bifidobacteria. In addition to the beneficial effects of probiotics, prebiotics and synbiotics on the physiology of pigs, the evidence of their efficacy against intestinal pathogens such as *E. coli* (Genovese *et al*., 2000; Huang *et al*., 2004; Konstantinov *et al*., 2008; Krause *et al*., 2010; Nemcova *et al*., 2007), *Campylobacter* (Jensen *et al*., 2012), *Brachyspira* (Hansen *et al*., 2011; Hansen *et al*., 2010; Thomsen *et al*., 2007) and *Salmonella* (Casey *et al*., 2007; Fedorka-Cray *et al*., 1999; Genovese *et al*., 2003) has been promising.

Numerous studies have demonstrated probiotics, prebiotic and synbiotics efficacy as briefly discussed above, however, some publications show conflicting

results. For example with regards to pathogen colonisation and shedding (Kreuzer *et al*., 2012; Martin-Pelaez *et al*., 2010; Szabo *et al*., 2009), whilst Taras *et al*. (2005) reported that although administration of probiotic *E. faecium* decreased the percentage of piglets with post weaning diarrhoea, no beneficial effect with regards to average daily weight gain or feed intake was observed. This could be due to the fact that multiple factors exist that might contribute to effectiveness of probiotics and prebiotic in particular those including their survival in the gut, dose, frequency of administration, specificity and the original health status of animals (Bomba *et al*., 2002).

1.6 Aims and objectives

The aforementioned introduction discusses the still existing problem of *S.* Typhimurium as an important zoonotic pathogen and that pigs and pork products might contribute to *Salmonella* persistence in the food chain. It also highlights the need for efficient pathogen control, including on farm alternative intervention strategies that will fill the niche since the EU ban of antimicrobial growth promoters. Thus the hypothesis to be tested in this PhD study is that the colonisation of the porcine gut by *S.* Typhimurium may be controlled by the combined suppressive effects of pre and probiotics.

Thus to fulfil this hypothesis, the specific aims and objectives of this project are:

- To screen and select prospective probiotic and prebiotic candidate to be used in the further studies as a part of a synbiotic combination.
- To study the effect of *L. plantarum* and lactulose on *S.* Typhimurium growth, and in mitigating against adhesion and invasion of this pathogen using porcine *in vitro* models.
- To improve understanding of the *L. plantarum* and lactulose mechanism of action by which the pathogen suppression is mediated.
- To investigate the effect of *L. plantarum* and lactulose on the porcine faecal microbiota, their fermentative activity and *S.* Typhimurium survival in the

porcine batch culture model.

- To investigate the ability of *L. plantarum* and lactulose to reduce pathogen colonisation and shedding in pigs experimentally challenged with *S.* Typhimurium – a pilot *in vivo* study.

Chapter 2

Materials and Methods

2.1 Bacteriological methods

2.1.1 Bacterial strains and culture conditions

All bacterial isolates used in these studies were obtained from the culture collection at the Animal Health and Veterinary Laboratories Agency (AHVLA, Weybridge, UK). A total of 11 *S.* Typhimurium isolates were provisionally used, however, following brief characterisation a nalidixic acid resistant derivative of *S.* Typhimurium SL1344 was used throughout the studies. In addition, a total of 16 *Lactobacillus* isolates and the control strains including *E. coli* DH5*α*-K12, *E. faecalis* ATCC 29212, *E. faecium* SF11770 and *C. jejuni* NCTC 11351 were used. *L. plantarum* B2028 (JC1) and *L. reuteri* B2026 were previously isolated from pig faeces (Collins *et al*., 2010).

Bacterial isolates were maintained in heart infusion broth (HIB) supplemented with 30% (v/v) glycerol (Oxoid) at -80°C. For routine use *Salmonella*, *E. coli* and *E. faecalis* ATCC 29212 isolates were cultured at 37°C for 16 hours, aerobically on 5% sheep's blood agar (SBA) or on lysogeny broth (also known as Luria-Bertani) (Bertani, 1951, 2004) agar without glucose (LB-G), respectively. LB-G broth cultures of both *Salmonella* and *E. coli* were incubated at 37°C for 16 hours, aerobically with gentle agitation (225 rpm). For studies where the *S.* Typhimurium SL1344 nal^r recovery was required from a microbially abundant samples brilliant green agar (BGA) (Kauffmann, 1935) containing 15 µg/ml nalidixic acid was used.

Lactobacilli were cultured on de Man, Rogosa, Sharpe (MRS) agar and broth (De Man *et al*., 1960) at 37°C for 24 hours, microaerophilically in GasPak jars using a GasPak™ plus system (BBL™) (94% H_2, 6% CO_2). Lactobacilli broth cultures (MRS broth) were incubated statically at 37°C for 24 hours. *C. jejuni* NCTC 11351 was cultured on 5% SBA, microaerophilically at 42°C.

2.1.2 Gram stain

Salmonella and lactobacilli isolates were cultured as described in section 2.1.1. A single colony was picked from the plate and smeared onto a glass slide. The smear was then heat fixed and 1% crystal violet (Sigma-Aldrich) applied to the smear for 60 seconds. The slide was washed with water after which Lugol's iodine (Sigma-Aldrich) was applied for 60 seconds and subsequently washed off with water. The excess of the crystal violet was removed using ethanol de-colouriser and the slide was once again washed using water. The counter stain safranin was applied for 60 seconds and again washed with water. Slides were allowed to air dry and then examined under oil immersion using light microscopy (Olympus CX21, × 1000). Gram stain results were recorded accordingly.

2.1.3 Catalase and oxidase test

Salmonella and lactobacilli isolates were cultured as described previously (section 2.1.1). For catalase testing, a representative colony of each isolate was picked and placed onto a Petri-dish and then a drop of 3% (v/v) hydrogen peroxide (H_2O_2) was mixed in with it. Production of gas (bubbles) was recorded as a catalase positive result. For oxidase testing, a representative colony of each isolate was picked from a plate with a sterile plastic inoculation loop and smeared onto an oxidase strip (Sigma-Aldrich). The result was read after 1 minute and development of a dark blue spot at the position of placed colony was recorded as a positive result. *C. jejuni* NCTC 11351was used as a positive control, whereas *E. faecium* SF11770 as a negative control.

2.1.4 API identification system

BioMerieux API 20E and API 50CH kit (BioMérieux) was used to determine bacterial species. Assays were conducted according to the manufacturer's instructions. *Salmonella* and *E. coli* isolates were tested using API 20E whereas lactobacilli were tested using API 50CH with specific API 50CHL medium.

For the API 20E kit, a saline suspension of a pure culture of *E. coli* and *Salmonella* strains was dispensed into mini cupules, rehydrating the medium present in each of the tubes. In addition, a number of tubes were overlaid with few drops of mineral oil to create anaerobic atmosphere (ADH, LDC, ODC, H_2S, URE). Strips were incubated at 37°C, aerobically for 18-24 hours and visual readings were taken and cross referenced with apiweb™ software (BioMérieux).

For API 50 CHL, lactobacilli were cultured on MRS agar as described previously in section 2.1.1 and subsequently colonies were removed from the plate and inoculated into API 50 CHL medium to obtain turbidity equal to McFarland 2.0. Tubes of the API strip were inoculated with prepared suspension, covered with few drops of mineral oil to create anaerobic conditions and incubated at 37°C for 48 hours. Visual readings were taken at 24 and 48 hours, subsequently cross referenced with apiweb™ software (BioMérieux).

2.1.5 Slide agglutination test

The identity of *Salmonella* was also confirmed using standard laboratory slide agglutinations. Agglutination tests were performed using *Salmonella* antisera (Pro-Lab Diagnostics) as per the manufacturer's instructions. Briefly, approximately 15 µl of anti-sera were placed upon a glass slide and then mixed with a small amount of bacterial suspension. The slide was gently tilted side to side and agglutination of the bacteria within one minute was considered a positive result. Irrelevant anti-sera acted as a negative control.

2.1.6 Hydrogen peroxide production

Production of H_2O_2 by lactobacilli isolates was assessed using the method described previously (Pascual *et al*., 2006). Briefly, a modified MRS was prepared supplemented with 0.01mg/ml horseradish peroxidase (Sigma-Aldrich) and 0.25 mg/ml 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) (Sigma-Aldrich). Isolates were sub-cultured on MRS medium containing TMB and incubated at 37°C for 72 hours in an anaerobic chamber. Following incubation plates were exposed to air for 30 minutes and the presence of blue colonies recorded as a positive result. Assays were performed on two separate occasions.

2.1.7 Colony overlay assay

A colony overlay assay was used according to Barbosa *et al*. (2005) to investigate antimicrobial activity of lactobacilli isolates against *S.* Typhimurium SL1344 nal^r growth. Briefly, three 5 µl volumes of an overnight culture (section 2.1.1) of each *Lactobacillus* isolate were inoculated onto MRS agar and incubated microaerophilically at 37°C for 24 hours. To kill the bacterial cells, plates were

exposed to chloroform (Sigma-Aldrich) vapours for 30 minutes followed by 20 minutes aeration. Subsequently, plates were overlaid with 0.7% (v/w) LB-G agar containing 10^5 cfu/ml of an overnight culture of the *S.* Typhimurium SL1344 nal^r. Plates were incubated aerobically at 37°C and zones of inhibition around the three spots were measured at 24 and 48 hours. MRS plates overlaid with the LB-G agar with or without indicator strain and without a probiotic were used as controls. Assays were performed on three separate occasions.

2.1.8 Conditioned medium assay

S. Typhimurium SL1344 nal^r and lactobacilli were cultured as described in section 2.1.1. To obtain lactobacilli cell free supernatants (CFSs) probiotic broth cultures were centrifuged (2447 × g) for 10 minutes at room temperature and supernatants were filter sterilized using 0.22 µm filter (Satorius Stedim Biotech).

For preliminary selection studies described in Chapter 3, an un-buffered CFS of sixteen lactobacilli was added (as 10% (v/v) dilution factor) to a 96-well micro-titre plate (Iwaki, SLS) inoculated with *S.* Typhimurium SL1344 nal^r culture (10^5 cfu/ml) in LB-G. Plates were incubated at 37°C and an optical density (OD) of 600nm was read every 15 minutes for 24 hours using FLUOstar OPTIMA (BMG LABTECH). *S.* Typhimurium SL1344 nal^r culture containing 10% (v/v) of MRS broth (pH 5.8) was used as a control. In addition LB-G broth with 10% (v/v) MRS and lactobacilli CFSs but without the indicator strain was included to standardise the OD readings.

For further studies described in Chapter 5, to identify mechanism of *L. plantarum* B2028 cell free supernatant inhibitory activity against *S.* Typhimurium SL1344 nal^r growth, CFS at pH 3.8 (un-buffered), adjusted to pH 4.5 and separately to pH 7.2 was tested as described above. The control broths were prepared accordingly; MRS broth adjusted to pH 3.8, 4.5 and 7.2 was used as a pH control, whereas MRS broth containing L-lactic acid (Sigma-Aldrich) and adjusted to pH 3.8, 4.5 and 7.2 was used as a lactic acid control (-LA) (Table 2.1). LB-G broth with 10% (v/v) of relevant MRS broth and *L. plantarum* B2028 CFS but without the indicator strain was included to standardise the OD readings.

In addition, for this assay and in subsequent studies where cell-free supernatant of *L. plantarum* B2028 was used as a part of synbiotic, for which the *L. plantarum* B2028 was cultured in modified MRS broth devoid of glucose but containing 1% (w/v) prebiotic lactulose, and hereinafter abbreviated as MRSL broth. Consequently, *L.*

plantarum B2028 cell free supernatant obtained from culture in MRSL broth was named CFSL. Growth inhibition assays of *S.* Typhimurium SL1344 nal^r were carried out subsequently as described above (Table 2.1).

Table 2.1 Experimental conditions used in conditioned medium assays.

Medium/Condition	Experimental strategy
LB-G	*S*. Typhimurium SL1344 nal^r culture medium
MRS	Standard lactobacilli growth medium containing 2% (w/v) glucose
MRSL	Modified lactobacilli growth medium devoid of glucose, containing 1% (w/v) lactulose
CFS	*L. plantarum* B2028 cell free supernatant obtained by centrifugation and filter sterilization of 24 h culture in MRS broth
CFSL	*L. plantarum* B2028 cell free supernatant obtained by centrifugation and filter sterilization of 24 h culture in MRSL broth
SL	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml)
MRS pH 3.8/ MRSL pH 3.8	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) MRS or MRSL broth, adjusted to pH 3.8
MRS pH 4.5/ MRSL pH 4.5	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) MRS or MRSL broth, adjusted to pH 4.5
MRS pH 7.2/ MRSL pH 7.2	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) MRS or MRSL broth, adjusted to pH 7.2
MRS-LA pH 3.8/ MRSL-LA pH 3.8	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) MRS or MRSL broth containing lactic acid, adjusted to pH 3.8
MRS-LA pH 4.5/ MRSL-LA pH 4.5	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) MRS or MRSL broth containing lactic acid, adjusted to pH 4.5
MRS-LA pH 7.2/ MRSL-LA pH 7.2	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) MRS or MRSL broth containing lactic acid, adjusted to pH 7.2
CFS pH 3.8/ CFSL pH 3.8	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) *L. plantarum* CSF or CFSL, at pH 3.8
CFS pH 4.5/ CFSL pH 4.5	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) *L. plantarum* CSF or CFSL, adjusted to pH 4.5
CFS pH 7.2/ CFSL pH 7.2	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) *L. plantarum* CSF or CFSL, adjusted to pH 7.2

2.1.9 Carbohydrate growth assay

Evaluation of *S.* Typhimurium SL1344 nal^r growth was monitored in minimal medium (MM) and standard LB-G medium supplemented accordingly with 1% (w/v)

of lactulose ((LAC, 4-*O*-β-D-galactopyranosyl- D-fructose) (Sigma-Aldrich)), FOS (Raftilose® P95, Tienen, Belgium) and GOS mixture (Bimuno®, Clasado). Control medium was included containing 1% (w/v) glucose. MM or LB-G medium containing respective carbohydrates was added to standardise the OD readings. For the assay *S.* Typhimurium SL1344 nal^r was cultured as described in section 2.1.1 in LB-G, centrifuged for 10 minutes and supernatant decanted. The pellet was washed in 0.1M PBS (pH 7.2) and resuspended at 1:1000 dilution in the respective test medium. For each condition, 200 µl of the re-suspended culture was dispensed into a 96-well micro-titre plate (Iwaki, SLS) and respective un-inoculated test medium was included as a blank standard for each condition. Plates were incubated at 37°C an optical density (OD) of 600nm was read every 15 minutes for 24 hours using FLUOstar OPTIMA (BMG LABTECH). Assays were conducted in triplicate on three separate occasions.
For analysis the mean OD values were used and area under the curve (AUC) calculated using GraphPad Prism® version 5 program (GraphPad Software, LaJolla, CA, USA). In addition the growth response on each prebiotic was calculated as described below.

Lactobacillus isolates ability to utilise prebiotics was tested subsequently as described above in carbohydrate-free basal MRS medium (Saarela *et al*., 2003) consisting of the following components (g/l): peptone from casein (10.0), yeast extract (5.0), $K_2HPO_4 \times 3H_2O$ (2.0), Na-acetate$\times 3H_2O$ (5.0), $(NH_4)_3C_6H_5O_7 \times 2H_2O$ (2.0), $MgSO_4 \times 7H_2O$ (0.2), $MnSO_4 \times 3H_2O$ (0.05) and Tween 80 (1ml).
For analysis, in addition to AUC calculations the growth of particular *Lactobacillus* isolate on each prebiotic relative to its growth on glucose was calculated over 24 hours using method given below, according to Kneifel *et al*. (2000):

Growth response = (mean values on tested prebiotic calculated from all OD readings / mean OD values of the same isolate grown in the basal medium containing glucose) × 100%

Additionally, the specific growth rate for lactobacilli on each prebiotic was calculated using formula according to Saminathan *et al.* (2011):

Growth rate (μ) = ($\ln x - \ln x_0$) / ($t - t_0$)

where:

x and x_0 are absorbances measured within exponential phase of growth

t and t_0 respective to the absorbance times

2.1.10 Acid tolerance

The acid tolerance test was conducted as described previously (Hyronimus *et al*., 2000) with slight modifications. *L. plantarum* B2028 was cultured in MRS broth, centrifuged at 2447 x g for 10 minutes and supernatant decanted. The pellet was washed in 0.1M PBS (pH 7.2) and the culture subsequently diluted (1:1000) in 0.1M PBS adjusted to pH 2.0, 2.5, 3.0 and non-adjusted PBS (pH 7.2) as a control. Samples were incubated microaerophilically at 37°C for 3 and 6 hours. Viable counts of bacteria were determined using Miles and Misra method (Miles *et al*., 1983), serially diluted culture was plated onto MRS agar. Plates were incubated for 48 hours and counted. The log reduction was calculated as shown below:

$$\text{Log reduction} = \log_{10} N_0 - \log_{10} N_t$$

where:

N_0 – number of viable cells at time point 0

N_t – number of viable cells at respective time after treatment

2.1.11 Bile tolerance

The ability of the probiotic candidate *L. plantarum* B2028 to survive in the presence of bile was assessed using Oxgall bile salt (Sigma-Aldrich) and freshly collected native porcine bile, using method adapted from Gilliland *et al*. (1984) with slight modifications. Porcine bile was collected from the gall bladder of a healthy pig, filter sterilized and used in concentrations as described further. Briefly, for the bile tolerance assay *L. plantarum* B2028 was cultured in MRS broth as described previously in section 2.1.1. Subsequently, MRS broth containing 0.3% or 0.6% (w/v) oxgall and 0.3%, 0.6% and 0.9% (v/v) porcine bile was inoculated with 10^6 cfu/ml of test isolate. MRS inoculated with *L. plantarum* B2028 culture but without addition of bile was used as positive growth control. For each condition, 200 µl of the re-suspended culture was transferred into a 96-well micro-titre plate (Iwaki, SLS) and respective un-inoculated MRS broth was included as a blank standard for each condition. Plates were incubated at 37°C an optical density (OD) of 600nm was read every 15 minutes for 24 hours using FLUOstar OPTIMA (BMG LABTECH). Assays were conducted in triplicate on three separate occasions.

Growth curves were plotted and analysis followed the principles described by Chateau *et al*. (1994) and based on the time necessary for each condition to reach an 0.3 unit at 600nm, and the difference (delay of growth) (*d*) between the growth control

and test conditions. This delay of growth (minutes) was used as a measure of the inhibitory effect of bile on tested isolate and to classify the isolates onto four following groups: resistant ($d \leq 15$ min), tolerant ($15 < d \leq 40$ min), weakly tolerant ($40 < d < 60$ min) and sensitive ($d \geq 60$ min).

2.1.12 Bile Salt Hydrolase (BSH) activity

BSH activity of the *L. plantarum* B2028 was evaluated using the method described by du Toit *et al*. (1998). Modified MRS plates were prepared by adding 0.5% (w/v) of taurodeoxycholic acid (TCDA), (Sigma-Aldrich) and 0.37g/l $CaCl_2$ to MRS agar. To perform the assays sterile filter disks were impregnated in an overnight culture of *L. plantarum* and placed onto the MRS plates. An MRS agar plate without TCDA supplementation was used as a control. The plates were incubated anaerobically at 37°C for 72 hours. Positive result for BSH activity was recorded when the taurodeoxycholic acid precipitated in the agar medium below and around the disc. The test was performed in triplicate on three separate occasions.

2.1.13 Antimicrobial susceptibility testing

For determining the minimum inhibitory concentration (MIC) of various antimicrobials for *L. plantarum* B2028 the broth microdilution method was used as recommended by EFSA (2008). For MICs LAB susceptibility test medium (LSM) consisting of a mixture of 90% Iso-Sensitest (ITS) broth and 10% MRS broth (pH 6.7) was used, as it fully supports lactobacilli growth and has no interaction with the antimicrobials tested (Klare *et al*., 2005; Klare *et al*., 2007). The following antimicrobials were obtained from Sigma-Aldrich and tested in the concentration ranges (ng/ µl): ampicillin, gentamicin, erythromycin and clindamycin (0.06-128) and streptomycin, kanamycin, chloramphenicol, tetracycline and vancomycin (1-256). Stock solutions of the antibiotics were prepared at twice the final concentration and filtered through 0.22 µm filter. Double strength concentration of the antibiotic was added to the first well and then the solution was double diluted into LSM medium. The control strain used for this assay was *E. faecalis* ATCC 29212 and for this strain the MIC's breakpoints were recommended by British Society for Antimicrobial Chemotherapy (BSAC). Antibiotic suspensions without the cell suspension were included as a negative control.

Briefly, *L. plantarum* B2028 was grown on MRS agar, whereas *E. faecalis* ATCC

29212 was grown on LB-G agar. Subsequently, fresh bacterial cells were taken off a plate using a sterile cotton swab and transferred to approximately 3 ml of 0.1M PBS (pH 7.2) to obtain turbidity equal to McFarland 1.0. A further 10 µl of the cell suspension was added to a volume of 1 ml in 24-well plate (Nunc) containing the test antibiotic dilutions. Plates were incubated at 37°C for 24 hours and MICs were recorded as the lowest antibiotic concentration to inhibit growth of the test organism. The assay was performed in duplicate on two separate occasions.

2.1.14 Porcine batch culture fermentation system

Batch culture fermentation system was set up according to previously published methodology (Sarbini *et al*., 2011; Saulnier *et al*., 2008) with slight modifications to mimic the conditions of a porcine distal colon (Martin-Pelaez *et al*., 2008). Two experiments were carried out, firstly for studies described in Chapter 3, system was inoculated with each of the following the substrates: lactulose (LAC) (Sigma-Aldrich) and FOS (Raftilose® P95, Tienen, Belgium). Where synbiotic was included, *L. plantarum* B2028 was used in addition to each prebiotic. In subsequent studies described in Chapter 5, batch culture system was inoculated with *S.* Typhimurium SL1344nal^r in addition to *L. plantarum* B2028 and LAC. To prepare porcine faecal slurry, porcine faecal samples were collected from *Salmonella*-free pigs (confirmed by bacteriology) (AHVLA) and immediately placed in an anaerobic jar and the samples were transported to the lab and used within maximum of 1-2 hours. Faecal samples were diluted with 0.1M PBS (pH 7.2) and homogenized in a stomacher (Stomacher 400, Seward) to yield 10 % (w/v) faecal slurry. Porcine faecal samples were confirmed to be *Salmonella*-free by direct plating out of serial dilutions onto BGA and further enrichment (see section 2.6.5).

Briefly, sterile stirred batch culture fermentation vessels were assembled and aseptically filled with sterile basal nutrient medium (135 ml each). The basal medium consisted of the following components (g/l): peptone water (2.0), yeast extract (2.0), NaCl (0.1), K_2HPO_4 (0.04), $MgSO_47H_20$ (0.04), $CaCl_26H_20$ (0.01), Tween 80 (1 ml), $NaHC0_3$ (2.0), hemin (0.05), vitamin K (10 µl), cysteine HCl (0.5), bile salts (0.5). The medium was gassed overnight with oxygen-free nitrogen (15 ml/min). Subsequently, glass vessels were inoculated with 15 ml of freshly prepared porcine faecal slurry (see above) and for preliminary studies appropriately labeled vessels were consequently inoculated with 1% (w/v) of respective prebiotic (LAC and FOS) and in vessels with

the synbiotic combination, an overnight culture (approx. 10^9 cfu/ml) of *L. plantarum* B2028 was added 1% (v/v). Vessel containing no pre or synbiotic was included as a control. The temperature in the vessels was maintained at the 37°C using a circulating water bath and to mimic conditions of a pig's large intestine, the pH was maintained between the ranges 6.4 - 6.6 using 0.5M NaOH and HCl.

Samples (3 ml from each vessel) were collected at time points: 0, 5, 10 and 24 hours post inoculation and stored accordingly for further analysis, namely for fluorescent *in situ* hybridisation (FISH) and for lactic acid and short chain fatty acids (SCFA) analysis. For FISH analysis, samples (375 µl) were fixed in (1125 µl) of ice-cold 4% (w/v) paraformaldehyde for 4-6 hours (4°C). Subsequently, samples were centrifuged at 10 000 × g for 5 minutes, washed twice with 0.1M PBS (pH 7.2) and the pellet was resuspended in 300 µl PBS/ethanol (50:50). Samples were thoroughly mixed by vortexing and stored at -20°C until required. For SCFA collected from each time point, samples (1 ml) were centrifuged at 10 000 × g for 10 minutes, supernatants were filter sterilized using 0.22 µm filter (Satorius Stedim Biotech) and stored at -20°C until required. The pellets were resuspended in PBS/glycerol (50:50) and stored at -20°C until required. All samples were prepared in duplicates.

During the subsequent study, an appropriately prepared system (see above) was inoculated with 1% (v/v) of an overnight culture (approx. 10^9 cfu/ml) of *S.* Typhimurium SL1344nal^r and in addition each vessel was inoculated with LAC, *L. plantarum* B2028, *L. plantarum* B2028 and LAC as described above. Assays were performed in duplicate on two separate occasions.

2.2 Molecular biology methods

2.2.1 DNA extraction

2.2.1.1 CTAB method

The cetyltrimethylammonium bromide (CTAB) method was used to obtain genomic DNA from pure cultures of lactobacilli and *Salmonella*. All reagents unless indicated otherwise were purchased from Sigma-Aldrich. *Salmonella* and lactobacilli culture was grown in LB-G and MRS broth respectively. Bacterial pellets were prepared by growth in broth culture followed by centrifugation at 4000 × g for 10 minutes, washing in 0.1M PBS (pH 7.2) and resuspension in 400 µl 1x Tris-ethylene-diamine tetra-acetic acid (EDTA) (TE) buffer (pH 8.0). In circumstances when DNA

was isolated from bacterial growth on agar plates, a bacterial suspension was prepared in 0.1M PBS (pH 7.2) and the bacterial pellet prepared as described above. An additional step was included for DNA isolation from lactobacilli with lysozyme, 20 mg/ml, added to TE buffer and incubated at 37°C for 60 minutes. Subsequently, the cells were lysed by the addition of 70 µl 10% (w/v) sodium dodecyl sulphate (SDS) and 70 µl of 20 mg/ml proteinase K and incubation at 55°C for 60 minutes. RNase (10 mg/ml) was added to remove RNA and samples were incubated at 37°C for 30 minutes. Subsequently, 100 µl 5 M NaCl and 80 µl of pre-warmed CTAB in 0.7 M NaCl were added and samples were incubated at 55°C for further 10 minutes. Following incubation, 750 µl chloroform/isoamyl alcohol (24:1) was added and samples were centrifuged at (16 000 × g) for 5 minutes. The aqueous phase was gently removed and transferred to clean phase lock tube (Eppendorf) and the wash step was repeated twice. Finally, the upper aqueous phase was transferred to a sterile microcentrifuge tube and 400 µl isopropanol was added to precipitate the DNA. Samples were left at -20°C for a minimum of 30 minutes and then centrifuged for 10 minutes (1600 × g) and the DNA pellet was washed with 500 µl 70% cold ethanol. Samples were subsequently centrifuged and supernatant discarded. The DNA pellet was air-dried and resuspended in 50 µl nuclease-free water (Ambion). DNA quantity and purity was determined using a NanoDrop™ spectrophotometer (Thermo Scientific) and 5 µl of the sample was run on 1% agarose gel for visualisation after EtBr staining. Aliquots were stored at - 20°C.

2.2.1.2 Bacterial DNA extraction from porcine batch culture samples

Bacterial DNA from porcine batch culture samples was extracted using a method provided by Dr Adele Costabile, University of Reading (personal communication). All reagents unless indicated otherwise were purchased from Sigma-Aldrich. Following collection, batch culture samples were centrifuged (16 000 × g) for 10 minutes and obtained pellet was stored in 50% (v/v) glycerol/0.1M PBS (pH 7.2) suspension at -20°C until DNA extraction was performed. Cells were harvested from the suspension at 16 000 × g for 5 minutes, washed once with 0.1M PBS (pH 7.2) and centrifuged again. Subsequently, the pellet was resuspended in 500 µl of TES buffer (pH 8.0) containing 8 µl 10 mg/ml lysozyme and 2 µl 1mg/ml mutanolysin and incubated at 37°C for 30 minutes. A 10 µl proteinase K (20 mg/ml) and 10 µl RNase (10 mg/ml) was added and the suspension was incubated at 65°C for 60 minutes. Then, 100 µl

10% (w/v) SDS was added to the samples and incubated for further 15 minutes after which samples were placed on ice for a minimum of 30 minutes. Subsequently, 620 µl phenol/chloroform/water (Applied Biosciences) was added to each sample, contents were mixed by inversion for at least 2 minutes and centrifuged at 4000 × g for 10 minutes. The upper aqueous layer was transferred to a sterile microcentrifuge tube and 1 ml of ice-cold ethanol was added. The suspension was mixed by inversion and left at -20°C overnight. Samples were subsequently centrifuged and the supernatant discarded. The DNA pellet was air-dried and resuspended in 50 µl nuclease-free water (Ambion). DNA quantity and purity was determined as described above.

2.2.1.3 Bacterial DNA extraction from porcine faecal samples

Freshly collected porcine faecal samples were stored at -20°C until further analysis. After thawing at room temperature, bacterial DNA was extracted using the QIamp DNA Stool Mini Kit (Qiagen, UK) according to the manufacturer's instructions. All reagents unless indicated otherwise were included in the extraction kit. Briefly, for the extraction 180-220 mg faecal sample was used, immediately mixed with ASL buffer and incubated at 90°C for 5 minutes. Following the lysis step, samples were incubated with the addition of lysozyme (20 mg/ml) (Sigma-Aldrich) at 37°C for 60 minutes. Subsequently, samples were centrifuged at 16 000 × g for 60 seconds and supernatant was transferred to a new microcentrifuge tube. To remove PCR inhibitors present within the sample, an InhibitiEX tablet was added to each of the samples and treated according to the manufactures instructions. The supernatant was transferred to a new microcentrifuge tube containing proteinase K, subsequently lysis buffer AL was added, vortexed and incubated at 70°C for 10 minutes. Ethanol (100 % (v/v)) was added to the lysate, applied to Qiamp spin column and centrifuged (16 000 × g) for 60 seconds after which the spin column was placed in a new collection tube. Following a series of washes using buffer AW1 and AW2 the spin column was transferred to a new microcentrifuge tube and the bound DNA was eluted using 50 µl of an elution buffer AE. DNA quantity and purity was determined as described above.

2.2.2 Polymerase Chain Reaction (PCR)

2.2.2.1 Standard PCR

Amplification of a target DNA sequence was performed by PCR (Saiki *et al*.,

1988) with a final reaction volume of 50 µl. The PCR reaction consisted of 25 µl HotStarTaq® DNA polymerase Master Mix (Qiagen), 1 µl of each forward and reverse primer (20 pmol/µl) (Sigma-Aldrich), 1 µl of genomic DNA (20-50 ng/µl) and made up to 50 µl using distilled water. PCR amplifications were performed using GeneAmp® PCR system 9700 thermal cycler (Applied Biosystems) and as follows: 1 cycle at 95°C for 15 minutes (initial denaturation), then 30 cycles consisting of denaturation at 95°C for 60 seconds, annealing (50-65°C) for 60 seconds, extension at 72°C for 60 seconds and final extension at 72°C for 10 minutes.

2.2.2.2 Multiplex PCR

Multiplex PCR was used for *Lactobacillus* species identification according to Kwon *et al*. (2004). Briefly, the PCR reaction with the final volume of 50 µl consisted of 25 µl HotStarTaq® DNA polymerase Master Mix (Qiagen), primers as outlined in a Table 2.2 (20 pmol each), genomic DNA template (20-50 ng/µl) and distilled water up to the final volume. PCR amplifications were performed on a thermal cycler (see above) and as follows: 1 cycle at 95°C for 15 minutes (initial denaturation), then 35 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 7 minutes.

2.2.3 PCR product purification

PCR products were purified using QIAquick® purification kit (Qiagen) according to the manufacturer's instructions. All reagents unless indicated otherwise were included in the extraction kit. Briefly, five volumes of the binding buffer PB were added to one volume of the PCR product and transferred to a QIAquick spin column. The column was centrifuged (16 000 × g) for 60 seconds and the flow-through was discarded. Following a wash step using the PE buffer to remove salts, column was placed in a clean microcentrifuge tube and DNA eluted with 30 µl EB buffer (elution buffer). If necessary, the PCR products were stored at -20°C until further use.

2.2.4 Gel electrophoresis

Agarose gel (1% or 2% (w/v)) was prepared in Tris-Acetate-EDTA buffer (TAE) (pH 8.0) (Sigma-Aldrich) and placed in Sub-Cell tank (Bio-Rad), sunken in TAE buffer. To each gel well, 5 µl sample and 1 µl DNA loading buffer (6×) (Promega) was loaded. A 1 Kb or 100 bp DNA molecular marker ladder (Promega) was run each time.

Running conditions were 75 volts for approximately 60 minutes. The gel was stained in 1µg/ml ethidium bromide solution (Sigma-Aldrich) for approximately 30 minutes and de-stained in distilled water. DNA was visualized under ultra-violet light using the GeneGenius gel imaging system.

Table 2.2 Multiplex PCR primes used in this studies (Kwon *et al*., 2004).

Target species	Primer	Sequence (5' to 3')	Target site[a]	Product (bp)[b]
Lactobacillus[c]	IDL03R	CCACCTTCCTCCGGTTTGTCA	1178-1198	-
Lactobacillus[c]	IDL04F	AGGGTGAAGTCGTAACAAGTAGCC	1499-1522	-
L.casei group[d]	IDL11F	TGGTCGGCAGAGTAACTGTTGTCG	427-495	727
L. acidophilus	IDL22R	AACTATCGCTTACGCTACCACTTTGC	2079-2104	606
L. delbrueckii	IDL31F	CTGTGCTACACCTAGAGATAGGTGG	1015-1039	184
L. gasseri	IDL42R	ATTTCAAGTTGAGTCTCTCTCTC	1748-1770	272
L. reuteri	IDL52R	ACCTGATTGACGATGGATCACCAGT	94-118	1105
L. plantarum	IDL62R	CTAGTGGTAACAGTTGATTAAAACTGC	1900-1926	428
L. rhamnosus	IDL73R	GCCAACAAGCTATGTGTTCGCTTGC	1922-1946	448

[a] All *Lactobacillus* - target site indicates the start and end point of the complimentary sequences annealing the forward and reverse primer, respectively.

[b] Approximated length of each PCR product derived from primer pair composed of species-specific primer and bacterial conserved primer (IDLC3R or IDLC4F).

[c] All *Lactobacillus* spp.

[d] *L. casei* group includes all of *L. casei*-related *Lactobacillus* species such as *L. casei* and *L. rhamnosus.*

Table adapted from Kwon *et al*. (2004).

2.2.5 16S rRNA gene sequencing

Lactobacillus DNA was extracted as described previously in section 2.2.1.1 and the 16S rRNA gene was amplified using PCR primers 63F and 1387R (Marchesi *et al*., 1998) (Table 2.3). The resulting PCR product (1324 bp) was visualized as described above and subsequently was sent to Central Sequencing Unit (CSU) (AHVLA, Weybridge, UK), where the sequencing was performed. The BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) was used according to the manufacturer's instructions and the sequencing was carried out on 3130*xl* Genetic Analyzer (Applied

Biosystems) with a 50 cm capillary length. The PCR parameters were as follows: 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 2 minutes.

Obtained from CSU sequence traces were analysed and edited in SeqMan (DNAstar, Lasergene) and the consensus sequences were saved in EditSeq (DNAstar, Lasergene). Subsequently, Basic Local Alignment Search Tool (BLAST) was used to carry out homology searches (Altschul *et al*., 1990) against National Centre for Biotechnology Information (NCBI) website.

Table 2.3 PCR primers used to amplify 1342 bp product of 16S rRNA gene (Marchesi *et al*., 1998).

Primer	**Sequence (5' to 3')**
63F	CAGGCCTA ACACATGCAAGTC
1387R	GGGCGGWGTGTACAAGGC

2.2.6 Gram-positive antimicrobial resistance array

To determine the presence antimicrobial resistance genes in the potential probiotic strain, the Indentibac AMR+ve™ microarray was employed (Perreten *et al*., 2005) and used according to the manufacturer's instructions. *L. plantarum* B2028 genomic DNA was isolated using the CTAB method. *E. faecium* SF11770 was used as the control for the Gram positive AMR. Genomic DNA was labeled using a randomly primed polymerization reaction (Bohlander *et al*., 1992; Perreten *et al*., 2005). PCR consisted of two rounds (A and B) and primers are showed in Table 2.4. Briefly, in round A the DNA (10-100 ng) was denatured, with Sequenase (5×) buffer and primer A (40 pmol) for 2 minutes at 94°C. Subsequently, during a cooling step at 10°C (5 minutes) mixture consisting of: (Sequenase buffer (5×), deoxyribonucleotide triphosphates dNTPs, dithiothreitol (DTT), bovine serum albumin (BSA), Sequenase and water was added to each sample. Samples were subjected to temperature ramping from 10°C to 37°C, at 37°C for 8 minutes, at 94°C for 2 minutes, at 10°C for 5 minutes. During this last step a Sequenase buffer (1:4 diluted) was added to the sample and again subjected to temperature ramping from 10°C to37°C and at 37°C for 8 minutes. The product from round A was mixed with sterile water and 7.5 µl was added to master mix (Quiagen buffer (10×), dNTPs, biotin-16-deoxyuridine triphosphate (dUTP), $MgCl_2$, primer B (100 pmol), Taq polymerase and water). Samples were amplified in thermal cycler

with the following conditions: 35 cycles of 94°C for 30 seconds, 40°C for 30 seconds, 50°C for 30 seconds, 72°C for 2 minutes, 4°C hold).

Table 2.4 Primers used in AMR+ve™ Gram positive microarray.

Primer	Sequence (5' to 3')
Primer A	GTTTCCCAGTCAGCATCNNNNNNNNN
Primer B	GTTTCCCAGTCACGATC

PCR product from round B was run on a 1% agarose gel to ensure successful amplification and 10 µl volume was used for the array. Briefly, before sample application the microarray tubes were washed with hybridization buffer and incubated at 30°C with shaking at 550 rpm for 5 minutes. A mix of PCR product and hybridization buffer was incubated at 95°C for 5 minutes, cooled down and transferred to the microarray tube. Tubes were incubated at 60°C with shaking at 550 rpm for 60 minutes and afterwards were washed three times with appropriate washing buffer. First wash was carried out at 30°C with shaking at 550 rpm for 5 minutes, and subsequent two washes at 20°C. Subsequently, microarray tubes were blocked with freshly prepared blocking solution (containing 2% (w/v) milk powder) at 30°C with shaking at 550 rpm for 15 minutes and diluted streptavidin-conjugated horseradish peroxidase (Poly-HRP Streptavidin) was added to the sample and incubated at 30°C for 15 minutes. This was then followed by a series of three washes at 30°C with shaking at and 550 rpm for 5 minutes. Subsequently, a 3',5,5'-tetramethylbenzidine (TMB) analog (SeramunGrün®, Seramun Diagnostica) was added to each array tube, incubated at the room temperature 10-15 minutes and the array was imaged using a tube reader (Clondiag). Data were analysed using Iconoclust software (Clondiag), which measures the signal intensity and the background for each spot on the array. AMR analyses were performed in duplicate.

2.2.7 Fluorescent *in situ* Hybridization

Fluorescent *in situ* Hybridization **(**FISH) using 16S rRNA oligonucleotide probes was performed accordingly to the previously described method (Daims *et al*., 2005). Specific probes (Table 2.5) for bifidobacteria, lactobacilli/enterococci and *Salmonella* were used to enumerate bacteria of interest. Total bacterial counts were obtained with

nucleic acid stain 4′6-diamidino-2-phenylindole (DAPI). Briefly, faecal slurry samples were collected from batch culture at 0, 5, 10 and 24 hours post inoculation and fixed in ice-cold 4% (w/v) paraformaldehyde for 4-6 hours at 4°C. Subsequently, samples were centrifuged at (16 000 × g) for 5 minutes, washed twice with 0.1M PBS (pH 7.2) and pellet was resuspended in 300 µl PBS/ethanol (50:50). Samples were mixed by vortexing and stored at -20°C until required. For hybridization, samples were diluted in PBS/SDS and a 20 µl aliquot of the diluted sample was pipetted onto Teflon- and poly-L-lysine- coated, six well slides (Tekdon Inc.). Samples were dried at 46°C for 15 minutes and then dehydrated in ethanol using 50%, 80% and 96% (v/v) concentrations (3 minutes in each). Following dehydration samples were again dried at 46°C to allow evaporation of alcohol. For the detection of lactobacilli/enterococci (probe Lab158), a 5 µl of lysozyme was applied to each well and left for 15 minutes at 37°C; washed briefly in water and dehydrated in a alcohol series as described above. A 50 µl mixture containing 5 µl of the probe and 45 µl of the hybridization buffer (HB) was applied onto each well and hybridization was performed for 4 hours using ISO20 oven (Grant Boekel). Following hybridization, slides were transferred into 50 ml wash buffer containing 20 µl DAPI and incubated in a water bath for 15 minutes. Slides were then washed in ice-cold water, dried under compressed air and 5 µl of anti-fade reagent was added to each well. The coverslips were applied and the slides were stored at 4°C until required. Slides were examined under an epifluoresce microscope (Eclipse 400; Nikon, Surrey, United Kingdom).

Bacteria were enumerated according to the following equation:

$$DF \times ACC \times 6732.42 \times 50 \times DF\ sample$$

where:

DF is the dilution factor, ACC is the average cells count of 15 fields of view, 6732.42 number is the area of the well divided by the area of the field of view, 50 is the factor that reverts the cell count to per milliliter of sample, DF sample is the dilution of sample according to the used probe or stain.

Table 2.5 16S rRNA oligonucleotide probes used for bacterial enumeration.

Probe name		**Sequence (5' to 3')**
Short	**Full name**	
Bif164[a]	S-G-Bif-0164-a-A-18	CATCCGGCATTACCACCC
Lab158[b†]	S-G-Lab-0158-a-A-20	GGTATTAGCAYCTGTTTCCA
Sal303[c]	L-S-Sal-1713-a-A-18	AATCACTTCACCTACGTG

[a] Target species: Most *Bifidobacterium* spp. and *Parascardovia dendicolens* (Langendijk *et al*., 1995)

[b] Target species: Most *Lactobacillus*, *Leuconostoc* and *Weissella* spp.; *Lactococcus lactis*; all *Vagococcus*, *Enterococcus*, *Melisococcus*, *Tetragenococcus*, *Catellicoccus*, *Pediococcus* and *Paralactobacillus* spp. (Harmsen *et al*., 1999).

[c] Target species: Different serovars of *Salmonella* spp. (Nordentoft *et al*., 1997).

† Requires lysozyme treatment.

Table adapted from Martin-Pealez *et al*. (2008).

2.3 Mass spectrometry methods

2.3.1 Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF)

Lactobacillus strains were grown on MRS agar as described in section 2.1.1 and fresh overnight culture was used for preparation of samples for MALDI ToF analysis. Method of Mellmann *et al*. (2008) was followed for sample preparation. Briefly, approximately 1 inoculation loop of fresh bacterial culture was resuspended in 300 µl water and 900 µl of ethanol. Samples were centrifuged at 13 000 × g and supernatant was decanted. To extract the cells, 50 µl of 70% formic acid (in water) was added to the bacterial pellet, sample was mixed thoroughly after which 50 µl of acetonitrile was added. Samples were again centrifuged at 13 000 × g for 2 minutes and 1 µl of obtained supernatant was transferred onto wells on a steel MALDI target plate. Supernatants were allowed to dry at room temperature and subsequently overlaid with 1.5 µl saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile-2.5% trifluoroacetic acid mixture (MALDI matrix) and samples were left to dry. Samples were analysed using Bruker Autoflex 2 MALDI-ToF machine (Bruker, Daltonics). The main spectral projection (MSP) spectra were processed with BioTyper software (Bruker, Daltonics) based on a comparison with the BioTyper reference library of MALDI-TOF mass spectra. Sample identification was based on two technical repeats.

2.4 Chromatography methods

2.4.1 High-performance liquid chromatography (HPLC)

SCFA analysis of lactobacilli CFS's and of batch culture samples were performed using an HPLC system and as described previously (Sarbini *et al*., 2011). Briefly, lactobacilli cultures and batch culture samples (section 2.1.14) were centrifuged for 10 minutes at (13 000 × g), supernatants were filter sterilized using 0.22 µm polycarbonate syringe filter and analysed using a HPLC system (LaChrom Merck Hitachi, Poole, Dorset, UK). System was equipped with a pump (L-7100), a refractive index detector (L-7490), and an autosampler (L-7200). For the separation of organic acids the ion-exclusion REZEX ROA-Organic acid column was used (Phenomenex Inc.). During the separation of organic acids the temperature was maintained at 85°C. The eluent used in the system was degassed sulphuric acid diluted in the HPLC-grade H_2O (0.0025 mmol/L); the flow rate was maintained at 0.5 ml/min. Standards for lactic, acetic, propionic, butyric, isobutyric, valeric and isovaleric acids were used at the following concentrations 12.5 mM, 25 mM, 50 mM, 75 mM, and 100 mM. Quantification of the samples was gained through calibration of the standards curves and results were the average from three replicates.

2.5 *In vitro* methods

2.5.1 Porcine intestinal epithelial cell line (IPEC-J2)

2.5.1.1 IPEC-J2 monolayers

IPEC-J2 cell line (derived from jejunum of an un-suckled 1-day old piglet) when used as monolayers were maintained according to Schierack *et al*. (2006). All reagents unless indicated otherwise were purchased from Sigma-Aldrich. Briefly, cells were cultured in a mixture of DMEM/Ham's F12 (1:1) medium supplemented with 5% (v/v) foetal calf serum (FCS), ITS media supplement (5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite), 5 ng/ml epidermal growth factor, 1% (v/v) non-essential amino acids, 1% (v/v) L-glutamine and gentamycin (50 µg/ml), hereafter referred as IPEC-J2 medium. Cells were maintained in a 5% CO_2 atmosphere.

For experiments cells were seeded in 24-well plates (Nunc) at a density of 2×10^5 cells per well and grown to confluent monolayers. During the assays cells were maintained in IPEC-J2 depleted of antibiotics.

2.5.1.2 Three-dimensional IPEC-J2 model

The porcine jejunal 3D cell culture model was based upon the previous methods, (Nickerson *et al*., 2001; Searle *et al*., 2010) and Dr J. W. Collins personal communication. For the 3D cell model standard IPEC-J2 medium was additionally supplemented with glucose, galactose and fructose (1 g/l, 0.25 g/l, 0.13 g/l) (Goodwin *et al*., 1993). IPEC-J2 monolayers were cultured as described above, in tissue culture flask. Cells were washed with Hank's balanced salt solution (HBSS) and detached using 0.25% trypsin for 5 minutes. Subsequently, fresh medium was added to remove trypsin, cells were centrifuged and then resuspended in a complete fresh IPEC-J2-3D medium to yield 2×10^5 cells/ml. Cell suspensions were mixed with 5mg/ml Cytodex™ microcarrier beads (Cytodex-3, size 133-225 µm) in a 50 ml Corning® centrifuge tube. Before use microcarrier beads were hydrated in 0.1M PBS (pH 7.2) and autoclaved according to supplier instructions. In order to facilitate the initial cell-bead attachment, suspensions were incubated at 37°C, 5% CO_2 for approximately 30 minutes. Subsequently, cell suspensions were introduced to the 50 ml rotating wall vessel (RWV) (Synthecon), air bubbles removed and cultured at a rotation starting at 13.7 rpm to maintain the cell-bead aggregates in the suspension. Cells were incubated for two days without media change to allow further attachment of cells. Circa 80% of the medium was replenished every second day and rotation was gradually increased over time as necessary. Cells were cultured in RWV (37°C, 5% CO_2) for 20-22 days. Following this time (day of the assay) cells were removed from the RWV, resuspended in IPEC-J medium (without antibiotics) to yield 5×10^5 cells/ml of suspension. To obtain the cell number in 1 ml, the cell aggregate suspensions were trypsinized and passed through a 70 µm sieve in order to separate them from the microcarrier beads. Following separation from the microcarrier bead fresh IPEC-J2 medium was added and cells were counted using a haemocytometer. For experiments cell aggregates were seeded into 1.5 ml centrifuge tubes.

2.5.2 *L. plantarum* B2028 and lactulose adherence assay.

Adhesion assay was performed as previously described (Dibb-Fuller *et al*., 1999) with slight modifications. Briefly, *L. plantarum* B2028 inocula were prepared by centrifugation (2447 × g) for 10 minutes, the supernatant was decanted, the pellet washed in 0.1M PBS (pH 7.2) and resuspended in IPEC-J2 medium to yield 5×10^7 cfu/ml. Cell-free supernatants were prepared by filter sterilization as described in

section 2.1.8. Cells were washed twice with Hank's balanced salt solution (HBSS) and 1 ml of the bacterial inoculum containing accordingly: *L. plantarum* B2028, *L. plantarum* B2028 + 1% (w/v) LAC, both cultured in standard MRS and *L. plantarum* B2028 cultured in MRSL (with 1% (w/v) LAC) was added to a 24-well plate. Wells containing IPEC-J2 medium only were included as a control conditions. Plates were incubated at 37°C and in the presence of 5% CO_2 for up to 2 hours. Subsequently, cells were washed three times with HBSS, disrupted with 1% Triton X-100 (Sigma-Aldrich) and mechanical stirring. Serially diluted cell suspensions ($10^0 – 10^6$) were plated onto MRS agar and incubated microaerophilically at 37°C for 24 hours.

2.5.3 *S.* Typhimurium viability and inhibition of invasion assay

To evaluate the effect of pre-treatment with *L. plantarum* B2028 supernatant on *S.* Typhimurium SL1344nal^r viability and invasion, a previously described method was followed (Coconnier *et al.*, 1997; Makras *et al.*, 2006). Briefly, *S.* Typhimurium SL1344nal^r cells (10^8 cfu/ml) were co-incubated with IPEC-J2 medium containing 10% (v/v) of *L. plantarum* B2028 cell-free CFS's (CFS/CFSL), pH control medium (MRS/MRSL) and lactic acid control medium (MRS-LA/MRSL-LA) at pH 3.8 and prepared as described previously in section 2.1.8. *S.* Typhimurium SL1344nal^r cells incubated in IPEC-J2 medium only and medium without bacterial inoculum added were included as controls. Following 1 hour incubation at 37°C serially diluted cell suspensions ($10^0 – 10^7$) were plated onto LB-G agar for enumeration. Subsequently, *S.* Typhimurium SL1344nal^r cells were washed in 0.1M PBS (pH 7.2) and resuspended in IPEC-J2 medium to yield 5×10^7 cfu/ml and the invasion assay was conducted as described below for competition assays (section 2.5.4).

2.5.4 Competition assays using IPEC-J2 monolayers and 3D model

Competition assays were performed as previously described (Dibb-Fuller *et al.*, 1999; Dibb-Fuller *et al.*, 2001; Mappley *et al.*, 2011; Searle *et al.*, 2009; Searle *et al.*, 2010). Briefly, *L. plantarum* B2028 and *S.* Typhimurium SL1344nal^r culture (section 2.1.1) was centrifuged (2447 × g) for 10 minutes and the supernatant was decanted. Bacterial pellet was washed in 0.1M PBS (pH 7.2) and subsequently resuspended in IPEC-J2 medium to yield 10^8 cfu/ml. Subsequently, bacterial inocula (Table 2.6) were added to 24-well plates (Nunc) (for monolayers) or 1.5 ml centrifuge tubes (for 3D cell aggregates) to a final concentration of 5×10^7 cfu in a well/tube in a 1 ml volume.

Wells/ centrifuge tubes containing IPEC-J2 medium only were included as a control conditions. Inoculated plates, one for adhesion and one for invasion and centrifuge tubes were incubated at 37°C (in the presence of 5% CO_2) for 60 minutes.

Following incubation, to count the *S.* Typhimurium SL1344nal^r associated, the cells were washed three times with HBSS and disrupted with 1% Triton X-100 (Sigma-Aldrich) using magnetic stirrer. To distinguish the number of *S.* Typhimurium SL1344nal^r invaded, the cells were washed twice with HBSS and inoculated with 1 ml of media containing 100 µg/ml gentamycin solution (Sigma-Aldrich) and incubated for additional 2 hours. Subsequently, cells were washed three times with HBSS and disrupted as described above. To determine the number of associated and invaded *S.* Typhimurium SL1344nal^r, 10-fold serial dilutions ($10^0 – 10^{-4}$) were plated onto LB-G agar (Oxoid). The number of adhered *S.* Typhimurium SL1344nal^r was obtained by subtracting the number of invaded bacteria from the number of associated bacteria. All assays were conducted in duplicate on three separate occasions.

Table 2.6 Experimental conditions used in the competition assays using IPEC-J2 cell line (monolayers and 3D cells) and porcine *in vitro* organ culture (IVOC) model.

Condition	Experimental strategy
SL	*S.* Typhimurium SL1344nal^r
MRSL pH 3.8	*S.* Typhimurium SL1344nal^r + 10% (v/v) MRSL[a] pH 3.8
LAC	*S.* Typhimurium SL1344nal^r + 1% (w/v) LAC
Lp	*S.* Typhimurium SL1344nal^r + *L. plantarum* B2028
Lp + LAC	*S.* Typhimurium SL1344nal^r + *L. plantarum* B2028 + 1% (w/v) LAC
Lp + CFSL	*S.* Typhimurium SL1344nal^r + *L. plantarum* B2028+ 10% (v/v) CFSL[b]
CFSL	*S.* Typhimurium SL1344nal^r + 10% (v/v) CFSL[b]

[a] MRS broth with 1% (w/v) lactulose as a main carbon source.

[b] Cell free supernatant of *L. plantarum* B2028 cultured in MRSL broth.

2.5.5 Giemsa assay

IPEC-J2 monolayers were used and maintained as described in section 2.5.1.1 with a slight modification as cells were grown in 24-well plates on 13 mm cover slips. Each well was inoculated with inocula containing *L. plantarum* B2028 cells, cells with 10% (v/v) CFS, or with 1% (w/v) LAC. Media only and media containing 10% (v/v) CFS and 1% (w/v) LAC were also added but depleted of bacterial cells. Plates were

incubated for 60 min as described for adhesion assays. Cells were stained with 10% Giemsa stain (Sigma-Aldrich) for 60 minutes and subsequently washed three times with distilled water and differentiated with 1% (v/v) acetic acid for 2 minutes followed by a wash with sterile distilled water. Cover slips containing cells were then removed from the 24-well plate and mounted on glass slides using DPX mountant (mixture of distyrene, a plasticizer and xylene) (Sigma-Aldrich). Slides were examined under oil immersion using light microscope (Olympus CX21, × 1000). Assays were performed in duplicate on two separate occasions.

2.5.6 Porcine *in vitro* organ culture

In vitro organ culture association assays were performed as previously described (Collins *et al*., 2010). Briefly, four 6-week old male commercial pigs were housed and fed a commercial un-medicated diet and water *ad libitum* for 7 days prior to the study. Pigs were euthanased by stunning and exanguination and subsequently placed on their dorsal recumbency and a mid-line incision was made. At *post-mortem* the whole intestinal tract was exteriorized and the jejunum and spiral colon located and sampled aseptically. Tissue samples were immediately placed in pre-cooled IVOC medium and transported to the laboratory on ice. The IVOC medium consisted of: complete RPMI 1640 medium containing 10% (v/v) fetal calf serum (FCS), 0.25% lactalbumin hydrosylate, 75 mM mercaptoethanol, 0.2 µg/ml hydrocortisone (1:1 chloroform/ethanol), 0.1 µg/ml insulin, 2 mM L-glutamine and L-aspartate (Collins *et al*., 2010; Girard *et al*., 2005). All reagents unless indicated otherwise were purchased from Sigma-Aldrich. Tissues were washed in IVOC medium, aseptically trimmed into *circa* 2cm x 2cm squares and immobilized in CellCrowns™ (Scaffdex) to obtain fixed mucosal surface of the tissue explants. Subsequently, immobilized intestinal explants from jejunum and colon were placed into pre-filled with IVOC medium (500 µl) 24-well plates (Nunc) with the mucosal surface facing upwards.

For association assays, *L. plantarum* B2028 and *S.* Typhimurium SL1344nal^{r} bacterial inocula were prepared and administered essentially as described for the competition assays (Table 2.6) with the exception that the final bacterial concentration in a well (1ml volume) was 10^{8} cfu. Plates were incubated at 37°C (in the presence of 5% CO_2) for 30 minutes with gentle agitation. Following the incubation tissue samples were washed in and placed in 9 ml 0.1M PBS (pH 7.2) and homogenized for bacteriological analysis. Serial dilutions (10^{0} – 10^{-6}) were plated out onto BGA

supplemented with 15 µg/ml nalidixic acid. Uninfected tissues were incubated with media only as controls. Assays were conducted in quadruplicate on two separate occasions.

2.6 *In vivo* methods

2.6.1 Animals and management

A total of twenty four, cross breed, mixed sex pig with a mean initial weight of 7.98 ± 0.7 kg were used for the study. Animals were weaned at 4 weeks of age, faecal samples were collected from sows (n = 3) and piglets and tested for the presence of *Salmonella* before the trial commencement. Pigs were randomly divided into four equal groups of six on the basis of their mean body weight and housed in a bio-containment facility (CLII). Each pen was equipped with a feeder and water supply from a water tray and from a nipple. Pens, feeders and water trays were cleaned on a daily basis. Pigs were fed commercial un-medicated pelleted feed (Lillico Attlee, Wm. Lillico & Son Ltd), according to their daily requirements (ASU Unit, AHVLA) and water was provided *ad libitum*. The left over feed was weighed every morning. Biosecurity measures were implemented in order to avoid cross contamination. These studies were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and were approved by the AHVLA ethics committee. All procedures were carried out at the AHVLA Coomblands Farm.

2.6.2 Bacterial strains preparation

S. Typhimurium SL1344 nal^r and *L. plantarum* B2028 were cultured as previously described in LB-G and MRS broth respectively, centrifuged (2447 × g) for 10 minutes and the supernatant was decanted. For *S*. Typhimurium SL1344 nal^r the pellet was and resuspended in 0.1M PBS (pH 7.2) to yield 10^9 cfu/ml bacteria and further diluted in sterile water to yield 5×10^7 cfu/ml. *L. plantrarum* B2028 was resuspended in sterile water and mixed with the small portion of feed for each pig to receive 10^{10} cfu/pig/day.

2.6.3 Experimental design and diets

Once distributed into four separate pens, the animals were allowed 7 days of acclimatisation after which the experimental diets were provided. The experimental treatment consisted of pelleted control diet without additives (Control), diet containing

1% (w/w) (15 ml/kg) lactulose given as a Duphalac syrup (LAC), diet containing 10^{10} cfu of *L. plantarum* B2028/animal/day (Lp) and diet containing 10^{10} cfu of *L. plantarum* B2028/animal/day together with 1% (w/v) (15 ml/kg) lactulose (Lp + LAC). Once stabilised on the above diets (6 days), each piglet in the all four groups was challenged with *S.* Typhimurium SL1344nal^r (5×10^8 cfu in 10 ml sterile water) administered by oral gavage (day 0). Approximately 30 minutes before the challenge animals were orally dosed with 10% (w/v) sodium bicarbonate solution to neutralise stomach acid (20 ml). Clinical performance was monitored throughout the entire study and samples were collected as detailed in section 2.6.4. At the end of the study, 10 days after *Salmonella* challenge all animals were humanely sacrificed for *post-mortem* examination.

2.6.4 Experimental monitoring and animals handling

All animals were examined clinically and scored as per Table 2.7 throughout the duration of the study. Feed consumption was recorded daily and body weights were taken weekly. Rectal temperature and faecal samples were collected from each piglet by rectal digital insertion on three separate occasions before *Salmonella* challenge, while animals were fed the experimental diet.

Table 2.7 Clinical score evaluation.

Clinical score	Diarrhoea	Feed consumption	Behaviour	Temperature
0	None	Normal	Active	Normal (38.5 – 40°C)
1+	Slight	Slightly off food	Active if stimulated	Raised (40.5 – 41°C)
2+	Watery (yellow, watery, +/- blood)	Off food	Inactive, when stimulated	Fever (above 41°C)

Following *S.* Typhimurium SL1344nal^r challenge, sampling was performed on a daily basis. Faecal samples (approx. 5 g) from each pen were collected from the floor daily and pooled for *Salmonella* testing.

2.6.5 Bacterial isolation and enumeration

Direct isolation and enumeration of *S.* Typhimurium SL1344nal^{r} from faeces were obtained by resuspending 10% (w/v) freshly collected samples in a buffered peptone water (BPW) (Edel and Kampelmacher, 1973), plating out serially diluted samples (10^{0}-10^{-5}) onto BGA supplemented with nalidixic acid 15 µg/ml and incubating at 37°C for 16 hours (Searle *et al*., 2009). For initial enrichment, resuspended in BPW samples were incubated at 37°C for 18 ± 2 hours. Subsequently, 100 µl of the overnight incubated broth was inoculated onto modified semisolid Rappaport-Vassiliadis medium (MSRV) (Aspinall *et al*., 1992; De Smedt *et al*., 1986) as a three equal drops. MSRV plates were incubated at 41.5°C for 24 hours. Plates which were negative for growth were allowed further 24 hours incubation and examined for growth. Subsequently, a 1 µl loop was used to pick the material from the edge of the definite growth, which was streaked onto Rambach agar and BGA agar supplemented with 15µg/ ml of nalidixic acid and colonies were allowed to develop for 24 hours at 37°C.

Tissues collected at *post-mortem*, were weighed (1 g where possible), homogenised in BPW (10% w/v) and serially diluted samples (10^{0}-10^{-5}) were plated onto BGA supplemented with 15µg/ ml of nalidixic acid. Samples were enriched as described above and a 100 µl aliquot was taken from the samples that were negative upon direct plating.

Lactobacilli numbers were determined by diluting fresh faecal samples 10% (w/v) in BPW and 10-fold serial dilutions in 0.1M PBS (pH 7.2) (10^{-1}-10^{-8}) were prepared and plated out on MRS agar (De Man *et al*., 1960). Plates were incubated at 37°C microaerophilically in GasPak jars using a GasPak™ plus system (BBL™) and colonies were allowed to develop for 48 hours. At four distinct time points during the study, feacal samples were subjected to PCR analysis to test for *L. plantarum* spp. presence (Kwon *et al*., 2004).

For *E. coli* and coliform enumeration of faeces, samples were prepared as above and plated onto a Chromagar ™ ECC (Randall *et al*., 2009). Plates were incubated at 37°C for 24 hours and all blue *E. coli* and mauve coliforms were counted.

2.6.6 *Post-mortem* examination

Ten days following pathogen challenge all pigs were euthanased by stunning and exanguination and subsequently placed in dorsal recumbency for a mid-line incision to

be made. Faecal samples were collected from rectum from all experimental animals. Tissues (jejunum, ileum, colon, caecum, rectum and mesenteric lymph nodes) from three pigs from each experimental group were sampled at *post-mortem* for bacteriology and histopathology.

2.7 Statistical analysis

Statistical analysis of the data presented within this thesis was performed using GraphPad Prism® version 5 program (GraphPad Software, LaJolla, CA, USA). Unless otherwise indicated the evaluation of statistically significant differences between results from the treatment groups and the treatment groups and the control were determined by repeated-measures One-way analysis of variance (ANOVA) and Bonferroni post test. For the studies where two factors (time and treatment) were taken into account, the repeated-measures Two-way ANOVA was used followed by the Bonferroni post test. The area under the curve (AUC) was calculated by the trapezoidal method with GraphPad Prism® version 5 program. The mean AUC values were compared using One-way ANOVA and Bonferroni post test. For analysis of *Salmonella* counts from the *in vivo* trial, the scoring system was applied and data between treated and control group compared.

Statistical significance was accepted at $P<0.05$ and differences among means with $0.05<P<0.10$ were accepted as tendencies to differences. All data within this thesis are presented as the mean with the standard error of the mean (SEM). The significance (*P* value) was visualized on graphs using an asterisk scale system (Table 2.8).

Table 2.8 *P* value summary.

***P* value**	**Symbol**
≤ 0.001	***
> 0.001 to ≤ 0.01	**
> 0.01 to ≤ 0.05	*
> 0.05	ns

Chapter 2

Materials and Methods

2.1 Bacteriological methods

2.1.1 Bacterial strains and culture conditions

All bacterial isolates used in these studies were obtained from the culture collection at the Animal Health and Veterinary Laboratories Agency (AHVLA, Weybridge, UK). A total of 11 *S.* Typhimurium isolates were provisionally used, however, following brief characterisation a nalidixic acid resistant derivative of *S.* Typhimurium SL1344 was used throughout the studies. In addition, a total of 16 *Lactobacillus* isolates and the control strains including *E. coli* DH5*α*-K12, *E. faecalis* ATCC 29212, *E. faecium* SF11770 and *C. jejuni* NCTC 11351 were used. *L. plantarum* B2028 (JC1) and *L. reuteri* B2026 were previously isolated from pig faeces (Collins *et al*., 2010).

Bacterial isolates were maintained in heart infusion broth (HIB) supplemented with 30% (v/v) glycerol (Oxoid) at -80°C. For routine use *Salmonella*, *E. coli* and *E. faecalis* ATCC 29212 isolates were cultured at 37°C for 16 hours, aerobically on 5% sheep's blood agar (SBA) or on lysogeny broth (also known as Luria-Bertani) (Bertani, 1951, 2004) agar without glucose (LB-G), respectively. LB-G broth cultures of both *Salmonella* and *E. coli* were incubated at 37°C for 16 hours, aerobically with gentle agitation (225 rpm). For studies where the *S.* Typhimurium SL1344 nal^r recovery was required from a microbially abundant samples brilliant green agar (BGA) (Kauffmann, 1935) containing 15 µg/ml nalidixic acid was used.

Lactobacilli were cultured on de Man, Rogosa, Sharpe (MRS) agar and broth (De Man *et al*., 1960) at 37°C for 24 hours, microaerophilically in GasPak jars using a GasPak™ plus system (BBL™) (94% H_2, 6% CO_2). Lactobacilli broth cultures (MRS broth) were incubated statically at 37°C for 24 hours. *C. jejuni* NCTC 11351 was cultured on 5% SBA, microaerophilically at 42°C.

2.1.2 Gram stain

Salmonella and lactobacilli isolates were cultured as described in section 2.1.1. A single colony was picked from the plate and smeared onto a glass slide. The smear was then heat fixed and 1% crystal violet (Sigma-Aldrich) applied to the smear for 60 seconds. The slide was washed with water after which Lugol's iodine (Sigma-Aldrich) was applied for 60 seconds and subsequently washed off with water. The excess of the crystal violet was removed using ethanol de-colouriser and the slide was once again washed using water. The counter stain safranin was applied for 60 seconds and again washed with water. Slides were allowed to air dry and then examined under oil immersion using light microscopy (Olympus CX21, × 1000). Gram stain results were recorded accordingly.

2.1.3 Catalase and oxidase test

Salmonella and lactobacilli isolates were cultured as described previously (section 2.1.1). For catalase testing, a representative colony of each isolate was picked and placed onto a Petri-dish and then a drop of 3% (v/v) hydrogen peroxide (H_2O_2) was mixed in with it. Production of gas (bubbles) was recorded as a catalase positive result. For oxidase testing, a representative colony of each isolate was picked from a plate with a sterile plastic inoculation loop and smeared onto an oxidase strip (Sigma-Aldrich). The result was read after 1 minute and development of a dark blue spot at the position of placed colony was recorded as a positive result. *C. jejuni* NCTC 11351was used as a positive control, whereas *E. faecium* SF11770 as a negative control.

2.1.4 API identification system

BioMerieux API 20E and API 50CH kit (BioMérieux) was used to determine bacterial species. Assays were conducted according to the manufacturer's instructions. *Salmonella* and *E. coli* isolates were tested using API 20E whereas lactobacilli were tested using API 50CH with specific API 50CHL medium.

For the API 20E kit, a saline suspension of a pure culture of *E. coli* and *Salmonella* strains was dispensed into mini cupules, rehydrating the medium present in each of the tubes. In addition, a number of tubes were overlaid with few drops of mineral oil to create anaerobic atmosphere (ADH, LDC, ODC, H_2S, URE). Strips were incubated at 37°C, aerobically for 18-24 hours and visual readings were taken and cross referenced with apiweb™ software (BioMérieux).

For API 50 CHL, lactobacilli were cultured on MRS agar as described previously in section 2.1.1 and subsequently colonies were removed from the plate and inoculated into API 50 CHL medium to obtain turbidity equal to McFarland 2.0. Tubes of the API strip were inoculated with prepared suspension, covered with few drops of mineral oil to create anaerobic conditions and incubated at 37°C for 48 hours. Visual readings were taken at 24 and 48 hours, subsequently cross referenced with apiweb™ software (BioMérieux).

2.1.5 Slide agglutination test

The identity of *Salmonella* was also confirmed using standard laboratory slide agglutinations. Agglutination tests were performed using *Salmonella* antisera (Pro-Lab Diagnostics) as per the manufacturer's instructions. Briefly, approximately 15 µl of anti-sera were placed upon a glass slide and then mixed with a small amount of bacterial suspension. The slide was gently tilted side to side and agglutination of the bacteria within one minute was considered a positive result. Irrelevant anti-sera acted as a negative control.

2.1.6 Hydrogen peroxide production

Production of H_2O_2 by lactobacilli isolates was assessed using the method described previously (Pascual *et al*., 2006). Briefly, a modified MRS was prepared supplemented with 0.01mg/ml horseradish peroxidase (Sigma-Aldrich) and 0.25 mg/ml 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) (Sigma-Aldrich). Isolates were sub-cultured on MRS medium containing TMB and incubated at 37°C for 72 hours in an anaerobic chamber. Following incubation plates were exposed to air for 30 minutes and the presence of blue colonies recorded as a positive result. Assays were performed on two separate occasions.

2.1.7 Colony overlay assay

A colony overlay assay was used according to Barbosa *et al*. (2005) to investigate antimicrobial activity of lactobacilli isolates against *S.* Typhimurium SL1344 nal^r growth. Briefly, three 5 µl volumes of an overnight culture (section 2.1.1) of each *Lactobacillus* isolate were inoculated onto MRS agar and incubated microaerophilically at 37°C for 24 hours. To kill the bacterial cells, plates were

exposed to chloroform (Sigma-Aldrich) vapours for 30 minutes followed by 20 minutes aeration. Subsequently, plates were overlaid with 0.7% (v/w) LB-G agar containing 10^5 cfu/ml of an overnight culture of the *S.* Typhimurium SL1344 nal^r. Plates were incubated aerobically at 37°C and zones of inhibition around the three spots were measured at 24 and 48 hours. MRS plates overlaid with the LB-G agar with or without indicator strain and without a probiotic were used as controls. Assays were performed on three separate occasions.

2.1.8 Conditioned medium assay

S. Typhimurium SL1344 nal^r and lactobacilli were cultured as described in section 2.1.1. To obtain lactobacilli cell free supernatants (CFSs) probiotic broth cultures were centrifuged (2447 × g) for 10 minutes at room temperature and supernatants were filter sterilized using 0.22 µm filter (Satorius Stedim Biotech).

For preliminary selection studies described in Chapter 3, an un-buffered CFS of sixteen lactobacilli was added (as 10% (v/v) dilution factor) to a 96-well micro-titre plate (Iwaki, SLS) inoculated with *S.* Typhimurium SL1344 nal^r culture (10^5 cfu/ml) in LB-G. Plates were incubated at 37°C and an optical density (OD) of 600nm was read every 15 minutes for 24 hours using FLUOstar OPTIMA (BMG LABTECH). *S.* Typhimurium SL1344 nal^r culture containing 10% (v/v) of MRS broth (pH 5.8) was used as a control. In addition LB-G broth with 10% (v/v) MRS and lactobacilli CFSs but without the indicator strain was included to standardise the OD readings.

For further studies described in Chapter 5, to identify mechanism of *L. plantarum* B2028 cell free supernatant inhibitory activity against *S.* Typhimurium SL1344 nal^r growth, CFS at pH 3.8 (un-buffered), adjusted to pH 4.5 and separately to pH 7.2 was tested as described above. The control broths were prepared accordingly; MRS broth adjusted to pH 3.8, 4.5 and 7.2 was used as a pH control, whereas MRS broth containing L-lactic acid (Sigma-Aldrich) and adjusted to pH 3.8, 4.5 and 7.2 was used as a lactic acid control (-LA) (Table 2.1). LB-G broth with 10% (v/v) of relevant MRS broth and *L. plantarum* B2028 CFS but without the indicator strain was included to standardise the OD readings.

In addition, for this assay and in subsequent studies where cell-free supernatant of *L. plantarum* B2028 was used as a part of synbiotic, for which the *L. plantarum* B2028 was cultured in modified MRS broth devoid of glucose but containing 1% (w/v) prebiotic lactulose, and hereinafter abbreviated as MRSL broth. Consequently, *L.*

plantarum B2028 cell free supernatant obtained from culture in MRSL broth was named CFSL. Growth inhibition assays of *S.* Typhimurium SL1344 nal^r were carried out subsequently as described above (Table 2.1).

Table 2.1 Experimental conditions used in conditioned medium assays.

Medium/Condition	Experimental strategy
LB-G	*S*. Typhimurium SL1344 nal^r culture medium
MRS	Standard lactobacilli growth medium containing 2% (w/v) glucose
MRSL	Modified lactobacilli growth medium devoid of glucose, containing 1% (w/v) lactulose
CFS	*L. plantarum* B2028 cell free supernatant obtained by centrifugation and filter sterilization of 24 h culture in MRS broth
CFSL	*L. plantarum* B2028 cell free supernatant obtained by centrifugation and filter sterilization of 24 h culture in MRSL broth
SL	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml)
MRS pH 3.8/ MRSL pH 3.8	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) MRS or MRSL broth, adjusted to pH 3.8
MRS pH 4.5/ MRSL pH 4.5	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) MRS or MRSL broth, adjusted to pH 4.5
MRS pH 7.2/ MRSL pH 7.2	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) MRS or MRSL broth, adjusted to pH 7.2
MRS-LA pH 3.8/ MRSL-LA pH 3.8	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) MRS or MRSL broth containing lactic acid, adjusted to pH 3.8
MRS-LA pH 4.5/ MRSL-LA pH 4.5	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) MRS or MRSL broth containing lactic acid, adjusted to pH 4.5
MRS-LA pH 7.2/ MRSL-LA pH 7.2	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) MRS or MRSL broth containing lactic acid, adjusted to pH 7.2
CFS pH 3.8/ CFSL pH 3.8	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) *L. plantarum* CSF or CFSL, at pH 3.8
CFS pH 4.5/ CFSL pH 4.5	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) *L. plantarum* CSF or CFSL, adjusted to pH 4.5
CFS pH 7.2/ CFSL pH 7.2	*S*. Typhimurium SL1344 nal^r culture in LB-G (10^5 cfu/ml) with 10% (v/v) *L. plantarum* CSF or CFSL, adjusted to pH 7.2

2.1.9 Carbohydrate growth assay

Evaluation of *S.* Typhimurium SL1344 nal^r growth was monitored in minimal medium (MM) and standard LB-G medium supplemented accordingly with 1% (w/v)

of lactulose ((LAC, 4-*O*-β-D-galactopyranosyl- D-fructose) (Sigma-Aldrich)), FOS (Raftilose® P95, Tienen, Belgium) and GOS mixture (Bimuno®, Clasado). Control medium was included containing 1% (w/v) glucose. MM or LB-G medium containing respective carbohydrates was added to standardise the OD readings. For the assay *S.* Typhimurium SL1344 nal^r was cultured as described in section 2.1.1 in LB-G, centrifuged for 10 minutes and supernatant decanted. The pellet was washed in 0.1M PBS (pH 7.2) and resuspended at 1:1000 dilution in the respective test medium. For each condition, 200 µl of the re-suspended culture was dispensed into a 96-well micro-titre plate (Iwaki, SLS) and respective un-inoculated test medium was included as a blank standard for each condition. Plates were incubated at 37°C an optical density (OD) of 600nm was read every 15 minutes for 24 hours using FLUOstar OPTIMA (BMG LABTECH). Assays were conducted in triplicate on three separate occasions.
For analysis the mean OD values were used and area under the curve (AUC) calculated using GraphPad Prism® version 5 program (GraphPad Software, LaJolla, CA, USA). In addition the growth response on each prebiotic was calculated as described below.

Lactobacillus isolates ability to utilise prebiotics was tested subsequently as described above in carbohydrate-free basal MRS medium (Saarela *et al*., 2003) consisting of the following components (g/l): peptone from casein (10.0), yeast extract (5.0), $K_2HPO_4 \times 3H_2O$ (2.0), Na-acetate$\times 3H_2O$ (5.0), $(NH_4)_3C_6H_5O_7 \times 2H_2O$ (2.0), $MgSO_4 \times 7H_2O$ (0.2), $MnSO_4 \times 3H_2O$ (0.05) and Tween 80 (1ml).
For analysis, in addition to AUC calculations the growth of particular *Lactobacillus* isolate on each prebiotic relative to its growth on glucose was calculated over 24 hours using method given below, according to Kneifel *et al*. (2000):

Growth response = (mean values on tested prebiotic calculated from all OD readings / mean OD values of the same isolate grown in the basal medium containing glucose) × 100%

Additionally, the specific growth rate for lactobacilli on each prebiotic was calculated using formula according to Saminathan *et al.* (2011):

Growth rate (μ) = ($\ln x - \ln x_0$) / ($t - t_0$)

where:

x and x_0 are absorbances measured within exponential phase of growth

t and t_0 respective to the absorbance times

2.1.10 Acid tolerance

The acid tolerance test was conducted as described previously (Hyronimus *et al*., 2000) with slight modifications. *L. plantarum* B2028 was cultured in MRS broth, centrifuged at 2447 x g for 10 minutes and supernatant decanted. The pellet was washed in 0.1M PBS (pH 7.2) and the culture subsequently diluted (1:1000) in 0.1M PBS adjusted to pH 2.0, 2.5, 3.0 and non-adjusted PBS (pH 7.2) as a control. Samples were incubated microaerophilically at 37°C for 3 and 6 hours. Viable counts of bacteria were determined using Miles and Misra method (Miles *et al*., 1983), serially diluted culture was plated onto MRS agar. Plates were incubated for 48 hours and counted. The log reduction was calculated as shown below:

$$\text{Log reduction} = \log_{10} N_0 - \log_{10} N_t$$

where:

N_0 – number of viable cells at time point 0

N_t – number of viable cells at respective time after treatment

2.1.11 Bile tolerance

The ability of the probiotic candidate *L. plantarum* B2028 to survive in the presence of bile was assessed using Oxgall bile salt (Sigma-Aldrich) and freshly collected native porcine bile, using method adapted from Gilliland *et al*. (1984) with slight modifications. Porcine bile was collected from the gall bladder of a healthy pig, filter sterilized and used in concentrations as described further. Briefly, for the bile tolerance assay *L. plantarum* B2028 was cultured in MRS broth as described previously in section 2.1.1. Subsequently, MRS broth containing 0.3% or 0.6% (w/v) oxgall and 0.3%, 0.6% and 0.9% (v/v) porcine bile was inoculated with 10^6 cfu/ml of test isolate. MRS inoculated with *L. plantarum* B2028 culture but without addition of bile was used as positive growth control. For each condition, 200 µl of the re-suspended culture was transferred into a 96-well micro-titre plate (Iwaki, SLS) and respective un-inoculated MRS broth was included as a blank standard for each condition. Plates were incubated at 37°C an optical density (OD) of 600nm was read every 15 minutes for 24 hours using FLUOstar OPTIMA (BMG LABTECH). Assays were conducted in triplicate on three separate occasions.

Growth curves were plotted and analysis followed the principles described by Chateau *et al*. (1994) and based on the time necessary for each condition to reach an 0.3 unit at 600nm, and the difference (delay of growth) (*d*) between the growth control

and test conditions. This delay of growth (minutes) was used as a measure of the inhibitory effect of bile on tested isolate and to classify the isolates onto four following groups: resistant ($d \leq 15$ min), tolerant ($15 < d \leq 40$ min), weakly tolerant ($40 < d < 60$ min) and sensitive ($d \geq 60$ min).

2.1.12 Bile Salt Hydrolase (BSH) activity

BSH activity of the *L. plantarum* B2028 was evaluated using the method described by du Toit *et al*. (1998). Modified MRS plates were prepared by adding 0.5% (w/v) of taurodeoxycholic acid (TCDA), (Sigma-Aldrich) and 0.37g/l $CaCl_2$ to MRS agar. To perform the assays sterile filter disks were impregnated in an overnight culture of *L. plantarum* and placed onto the MRS plates. An MRS agar plate without TCDA supplementation was used as a control. The plates were incubated anaerobically at 37°C for 72 hours. Positive result for BSH activity was recorded when the taurodeoxycholic acid precipitated in the agar medium below and around the disc. The test was performed in triplicate on three separate occasions.

2.1.13 Antimicrobial susceptibility testing

For determining the minimum inhibitory concentration (MIC) of various antimicrobials for *L. plantarum* B2028 the broth microdilution method was used as recommended by EFSA (2008). For MICs LAB susceptibility test medium (LSM) consisting of a mixture of 90% Iso-Sensitest (ITS) broth and 10% MRS broth (pH 6.7) was used, as it fully supports lactobacilli growth and has no interaction with the antimicrobials tested (Klare *et al*., 2005; Klare *et al*., 2007). The following antimicrobials were obtained from Sigma-Aldrich and tested in the concentration ranges (ng/ µl): ampicillin, gentamicin, erythromycin and clindamycin (0.06-128) and streptomycin, kanamycin, chloramphenicol, tetracycline and vancomycin (1-256). Stock solutions of the antibiotics were prepared at twice the final concentration and filtered through 0.22 µm filter. Double strength concentration of the antibiotic was added to the first well and then the solution was double diluted into LSM medium. The control strain used for this assay was *E. faecalis* ATCC 29212 and for this strain the MIC's breakpoints were recommended by British Society for Antimicrobial Chemotherapy (BSAC). Antibiotic suspensions without the cell suspension were included as a negative control.

Briefly, *L. plantarum* B2028 was grown on MRS agar, whereas *E. faecalis* ATCC

29212 was grown on LB-G agar. Subsequently, fresh bacterial cells were taken off a plate using a sterile cotton swab and transferred to approximately 3 ml of 0.1M PBS (pH 7.2) to obtain turbidity equal to McFarland 1.0. A further 10 µl of the cell suspension was added to a volume of 1 ml in 24-well plate (Nunc) containing the test antibiotic dilutions. Plates were incubated at 37°C for 24 hours and MICs were recorded as the lowest antibiotic concentration to inhibit growth of the test organism. The assay was performed in duplicate on two separate occasions.

2.1.14 Porcine batch culture fermentation system

Batch culture fermentation system was set up according to previously published methodology (Sarbini *et al*., 2011; Saulnier *et al*., 2008) with slight modifications to mimic the conditions of a porcine distal colon (Martin-Pelaez *et al*., 2008). Two experiments were carried out, firstly for studies described in Chapter 3, system was inoculated with each of the following the substrates: lactulose (LAC) (Sigma-Aldrich) and FOS (Raftilose® P95, Tienen, Belgium). Where synbiotic was included, *L. plantarum* B2028 was used in addition to each prebiotic. In subsequent studies described in Chapter 5, batch culture system was inoculated with *S.* Typhimurium SL1344nal^r in addition to *L. plantarum* B2028 and LAC. To prepare porcine faecal slurry, porcine faecal samples were collected from *Salmonella*-free pigs (confirmed by bacteriology) (AHVLA) and immediately placed in an anaerobic jar and the samples were transported to the lab and used within maximum of 1-2 hours. Faecal samples were diluted with 0.1M PBS (pH 7.2) and homogenized in a stomacher (Stomacher 400, Seward) to yield 10 % (w/v) faecal slurry. Porcine faecal samples were confirmed to be *Salmonella*-free by direct plating out of serial dilutions onto BGA and further enrichment (see section 2.6.5).

Briefly, sterile stirred batch culture fermentation vessels were assembled and aseptically filled with sterile basal nutrient medium (135 ml each). The basal medium consisted of the following components (g/l): peptone water (2.0), yeast extract (2.0), NaCl (0.1), K_2HPO_4 (0.04), $MgSO_47H_20$ (0.04), $CaCl_26H_20$ (0.01), Tween 80 (1 ml), $NaHC0_3$ (2.0), hemin (0.05), vitamin K (10 µl), cysteine HCl (0.5), bile salts (0.5). The medium was gassed overnight with oxygen-free nitrogen (15 ml/min). Subsequently, glass vessels were inoculated with 15 ml of freshly prepared porcine faecal slurry (see above) and for preliminary studies appropriately labeled vessels were consequently inoculated with 1% (w/v) of respective prebiotic (LAC and FOS) and in vessels with

the synbiotic combination, an overnight culture (approx. 10^9 cfu/ml) of *L. plantarum* B2028 was added 1% (v/v). Vessel containing no pre or synbiotic was included as a control. The temperature in the vessels was maintained at the 37°C using a circulating water bath and to mimic conditions of a pig's large intestine, the pH was maintained between the ranges 6.4 - 6.6 using 0.5M NaOH and HCl.

Samples (3 ml from each vessel) were collected at time points: 0, 5, 10 and 24 hours post inoculation and stored accordingly for further analysis, namely for fluorescent *in situ* hybridisation (FISH) and for lactic acid and short chain fatty acids (SCFA) analysis. For FISH analysis, samples (375 µl) were fixed in (1125 µl) of ice-cold 4% (w/v) paraformaldehyde for 4-6 hours (4°C). Subsequently, samples were centrifuged at 10 000 × g for 5 minutes, washed twice with 0.1M PBS (pH 7.2) and the pellet was resuspended in 300 µl PBS/ethanol (50:50). Samples were thoroughly mixed by vortexing and stored at -20°C until required. For SCFA collected from each time point, samples (1 ml) were centrifuged at 10 000 × g for 10 minutes, supernatants were filter sterilized using 0.22 µm filter (Satorius Stedim Biotech) and stored at -20°C until required. The pellets were resuspended in PBS/glycerol (50:50) and stored at -20°C until required. All samples were prepared in duplicates.

During the subsequent study, an appropriately prepared system (see above) was inoculated with 1% (v/v) of an overnight culture (approx. 10^9 cfu/ml) of *S.* Typhimurium SL1344nal^r and in addition each vessel was inoculated with LAC, *L. plantarum* B2028, *L. plantarum* B2028 and LAC as described above. Assays were performed in duplicate on two separate occasions.

2.2 Molecular biology methods

2.2.1 DNA extraction

2.2.1.1 CTAB method

The cetyltrimethylammonium bromide (CTAB) method was used to obtain genomic DNA from pure cultures of lactobacilli and *Salmonella*. All reagents unless indicated otherwise were purchased from Sigma-Aldrich. *Salmonella* and lactobacilli culture was grown in LB-G and MRS broth respectively. Bacterial pellets were prepared by growth in broth culture followed by centrifugation at 4000 × g for 10 minutes, washing in 0.1M PBS (pH 7.2) and resuspension in 400 µl 1x Tris-ethylene-diamine tetra-acetic acid (EDTA) (TE) buffer (pH 8.0). In circumstances when DNA

was isolated from bacterial growth on agar plates, a bacterial suspension was prepared in 0.1M PBS (pH 7.2) and the bacterial pellet prepared as described above. An additional step was included for DNA isolation from lactobacilli with lysozyme, 20 mg/ml, added to TE buffer and incubated at 37°C for 60 minutes. Subsequently, the cells were lysed by the addition of 70 µl 10% (w/v) sodium dodecyl sulphate (SDS) and 70 µl of 20 mg/ml proteinase K and incubation at 55°C for 60 minutes. RNase (10 mg/ml) was added to remove RNA and samples were incubated at 37°C for 30 minutes. Subsequently, 100 µl 5 M NaCl and 80 µl of pre-warmed CTAB in 0.7 M NaCl were added and samples were incubated at 55°C for further 10 minutes. Following incubation, 750 µl chloroform/isoamyl alcohol (24:1) was added and samples were centrifuged at (16 000 × g) for 5 minutes. The aqueous phase was gently removed and transferred to clean phase lock tube (Eppendorf) and the wash step was repeated twice. Finally, the upper aqueous phase was transferred to a sterile microcentrifuge tube and 400 µl isopropanol was added to precipitate the DNA. Samples were left at -20°C for a minimum of 30 minutes and then centrifuged for 10 minutes (1600 × g) and the DNA pellet was washed with 500 µl 70% cold ethanol. Samples were subsequently centrifuged and supernatant discarded. The DNA pellet was air-dried and resuspended in 50 µl nuclease-free water (Ambion). DNA quantity and purity was determined using a NanoDrop™ spectrophotometer (Thermo Scientific) and 5 µl of the sample was run on 1% agarose gel for visualisation after EtBr staining. Aliquots were stored at - 20°C.

2.2.1.2 Bacterial DNA extraction from porcine batch culture samples

Bacterial DNA from porcine batch culture samples was extracted using a method provided by Dr Adele Costabile, University of Reading (personal communication). All reagents unless indicated otherwise were purchased from Sigma-Aldrich. Following collection, batch culture samples were centrifuged (16 000 × g) for 10 minutes and obtained pellet was stored in 50% (v/v) glycerol/0.1M PBS (pH 7.2) suspension at -20°C until DNA extraction was performed. Cells were harvested from the suspension at 16 000 × g for 5 minutes, washed once with 0.1M PBS (pH 7.2) and centrifuged again. Subsequently, the pellet was resuspended in 500 µl of TES buffer (pH 8.0) containing 8 µl 10 mg/ml lysozyme and 2 µl 1mg/ml mutanolysin and incubated at 37°C for 30 minutes. A 10 µl proteinase K (20 mg/ml) and 10 µl RNase (10 mg/ml) was added and the suspension was incubated at 65°C for 60 minutes. Then, 100 µl

10% (w/v) SDS was added to the samples and incubated for further 15 minutes after which samples were placed on ice for a minimum of 30 minutes. Subsequently, 620 µl phenol/chloroform/water (Applied Biosciences) was added to each sample, contents were mixed by inversion for at least 2 minutes and centrifuged at 4000 × g for 10 minutes. The upper aqueous layer was transferred to a sterile microcentrifuge tube and 1 ml of ice-cold ethanol was added. The suspension was mixed by inversion and left at -20°C overnight. Samples were subsequently centrifuged and the supernatant discarded. The DNA pellet was air-dried and resuspended in 50 µl nuclease-free water (Ambion). DNA quantity and purity was determined as described above.

2.2.1.3 Bacterial DNA extraction from porcine faecal samples

Freshly collected porcine faecal samples were stored at -20°C until further analysis. After thawing at room temperature, bacterial DNA was extracted using the QIamp DNA Stool Mini Kit (Qiagen, UK) according to the manufacturer's instructions. All reagents unless indicated otherwise were included in the extraction kit. Briefly, for the extraction 180-220 mg faecal sample was used, immediately mixed with ASL buffer and incubated at 90°C for 5 minutes. Following the lysis step, samples were incubated with the addition of lysozyme (20 mg/ml) (Sigma-Aldrich) at 37°C for 60 minutes. Subsequently, samples were centrifuged at 16 000 × g for 60 seconds and supernatant was transferred to a new microcentrifuge tube. To remove PCR inhibitors present within the sample, an InhibitiEX tablet was added to each of the samples and treated according to the manufactures instructions. The supernatant was transferred to a new microcentrifuge tube containing proteinase K, subsequently lysis buffer AL was added, vortexed and incubated at 70°C for 10 minutes. Ethanol (100 % (v/v)) was added to the lysate, applied to Qiamp spin column and centrifuged (16 000 × g) for 60 seconds after which the spin column was placed in a new collection tube. Following a series of washes using buffer AW1 and AW2 the spin column was transferred to a new microcentrifuge tube and the bound DNA was eluted using 50 µl of an elution buffer AE. DNA quantity and purity was determined as described above.

2.2.2 Polymerase Chain Reaction (PCR)

2.2.2.1 Standard PCR

Amplification of a target DNA sequence was performed by PCR (Saiki *et al*.,

1988) with a final reaction volume of 50 µl. The PCR reaction consisted of 25 µl HotStarTaq® DNA polymerase Master Mix (Qiagen), 1 µl of each forward and reverse primer (20 pmol/µl) (Sigma-Aldrich), 1 µl of genomic DNA (20-50 ng/µl) and made up to 50 µl using distilled water. PCR amplifications were performed using GeneAmp® PCR system 9700 thermal cycler (Applied Biosystems) and as follows: 1 cycle at 95°C for 15 minutes (initial denaturation), then 30 cycles consisting of denaturation at 95°C for 60 seconds, annealing (50-65°C) for 60 seconds, extension at 72°C for 60 seconds and final extension at 72°C for 10 minutes.

2.2.2.2 Multiplex PCR

Multiplex PCR was used for *Lactobacillus* species identification according to Kwon *et al*. (2004). Briefly, the PCR reaction with the final volume of 50 µl consisted of 25 µl HotStarTaq® DNA polymerase Master Mix (Qiagen), primers as outlined in a Table 2.2 (20 pmol each), genomic DNA template (20-50 ng/µl) and distilled water up to the final volume. PCR amplifications were performed on a thermal cycler (see above) and as follows: 1 cycle at 95°C for 15 minutes (initial denaturation), then 35 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 7 minutes.

2.2.3 PCR product purification

PCR products were purified using QIAquick® purification kit (Qiagen) according to the manufacturer's instructions. All reagents unless indicated otherwise were included in the extraction kit. Briefly, five volumes of the binding buffer PB were added to one volume of the PCR product and transferred to a QIAquick spin column. The column was centrifuged (16 000 × g) for 60 seconds and the flow-through was discarded. Following a wash step using the PE buffer to remove salts, column was placed in a clean microcentrifuge tube and DNA eluted with 30 µl EB buffer (elution buffer). If necessary, the PCR products were stored at -20°C until further use.

2.2.4 Gel electrophoresis

Agarose gel (1% or 2% (w/v)) was prepared in Tris-Acetate-EDTA buffer (TAE) (pH 8.0) (Sigma-Aldrich) and placed in Sub-Cell tank (Bio-Rad), sunken in TAE buffer. To each gel well, 5 µl sample and 1 µl DNA loading buffer (6×) (Promega) was loaded. A 1 Kb or 100 bp DNA molecular marker ladder (Promega) was run each time.

Running conditions were 75 volts for approximately 60 minutes. The gel was stained in 1µg/ml ethidium bromide solution (Sigma-Aldrich) for approximately 30 minutes and de-stained in distilled water. DNA was visualized under ultra-violet light using the GeneGenius gel imaging system.

Table 2.2 Multiplex PCR primes used in this studies (Kwon *et al*., 2004).

Target species	Primer	Sequence (5' to 3')	Target site[a]	Product (bp)[b]
Lactobacillus[c]	IDL03R	CCACCTTCCTCCGGTTTGTCA	1178-1198	-
Lactobacillus[c]	IDL04F	AGGGTGAAGTCGTAACAAGTAGCC	1499-1522	-
L.casei group[d]	IDL11F	TGGTCGGCAGAGTAACTGTTGTCG	427-495	727
L. acidophilus	IDL22R	AACTATCGCTTACGCTACCACTTTGC	2079-2104	606
L. delbrueckii	IDL31F	CTGTGCTACACCTAGAGATAGGTGG	1015-1039	184
L. gasseri	IDL42R	ATTTCAAGTTGAGTCTCTCTCTC	1748-1770	272
L. reuteri	IDL52R	ACCTGATTGACGATGGATCACCAGT	94-118	1105
L. plantarum	IDL62R	CTAGTGGTAACAGTTGATTAAAACTGC	1900-1926	428
L. rhamnosus	IDL73R	GCCAACAAGCTATGTGTTCGCTTGC	1922-1946	448

[a] All *Lactobacillus* - target site indicates the start and end point of the complimentary sequences annealing the forward and reverse primer, respectively.

[b] Approximated length of each PCR product derived from primer pair composed of species-specific primer and bacterial conserved primer (IDLC3R or IDLC4F).

[c] All *Lactobacillus* spp.

[d] *L. casei* group includes all of *L. casei*-related *Lactobacillus* species such as *L. casei* and *L. rhamnosus.*

Table adapted from Kwon *et al*. (2004).

2.2.5 16S rRNA gene sequencing

Lactobacillus DNA was extracted as described previously in section 2.2.1.1 and the 16S rRNA gene was amplified using PCR primers 63F and 1387R (Marchesi *et al*., 1998) (Table 2.3). The resulting PCR product (1324 bp) was visualized as described above and subsequently was sent to Central Sequencing Unit (CSU) (AHVLA, Weybridge, UK), where the sequencing was performed. The BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) was used according to the manufacturer's instructions and the sequencing was carried out on 3130*xl* Genetic Analyzer (Applied

Biosystems) with a 50 cm capillary length. The PCR parameters were as follows: 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 2 minutes.

Obtained from CSU sequence traces were analysed and edited in SeqMan (DNAstar, Lasergene) and the consensus sequences were saved in EditSeq (DNAstar, Lasergene). Subsequently, Basic Local Alignment Search Tool (BLAST) was used to carry out homology searches (Altschul *et al*., 1990) against National Centre for Biotechnology Information (NCBI) website.

Table 2.3 PCR primers used to amplify 1342 bp product of 16S rRNA gene (Marchesi *et al*., 1998).

Primer	**Sequence (5' to 3')**
63F	CAGGCCTA ACACATGCAAGTC
1387R	GGGCGGWGTGTACAAGGC

2.2.6 Gram-positive antimicrobial resistance array

To determine the presence antimicrobial resistance genes in the potential probiotic strain, the Indentibac AMR+ve™ microarray was employed (Perreten *et al*., 2005) and used according to the manufacturer's instructions. *L. plantarum* B2028 genomic DNA was isolated using the CTAB method. *E. faecium* SF11770 was used as the control for the Gram positive AMR. Genomic DNA was labeled using a randomly primed polymerization reaction (Bohlander *et al*., 1992; Perreten *et al*., 2005). PCR consisted of two rounds (A and B) and primers are showed in Table 2.4. Briefly, in round A the DNA (10-100 ng) was denatured, with Sequenase (5×) buffer and primer A (40 pmol) for 2 minutes at 94°C. Subsequently, during a cooling step at 10°C (5 minutes) mixture consisting of: (Sequenase buffer (5×), deoxyribonucleotide triphosphates dNTPs, dithiothreitol (DTT), bovine serum albumin (BSA), Sequenase and water was added to each sample. Samples were subjected to temperature ramping from 10°C to 37°C, at 37°C for 8 minutes, at 94°C for 2 minutes, at 10°C for 5 minutes. During this last step a Sequenase buffer (1:4 diluted) was added to the sample and again subjected to temperature ramping from 10°C to37°C and at 37°C for 8 minutes. The product from round A was mixed with sterile water and 7.5 µl was added to master mix (Quiagen buffer (10×), dNTPs, biotin-16-deoxyuridine triphosphate (dUTP), $MgCl_2$, primer B (100 pmol), Taq polymerase and water). Samples were amplified in thermal cycler

with the following conditions: 35 cycles of 94°C for 30 seconds, 40°C for 30 seconds, 50°C for 30 seconds, 72°C for 2 minutes, 4°C hold).

Table 2.4 Primers used in AMR+ve™ Gram positive microarray.

Primer	Sequence (5' to 3')
Primer A	GTTTCCCAGTCAGCATCNNNNNNNNN
Primer B	GTTTCCCAGTCACGATC

PCR product from round B was run on a 1% agarose gel to ensure successful amplification and 10 µl volume was used for the array. Briefly, before sample application the microarray tubes were washed with hybridization buffer and incubated at 30°C with shaking at 550 rpm for 5 minutes. A mix of PCR product and hybridization buffer was incubated at 95°C for 5 minutes, cooled down and transferred to the microarray tube. Tubes were incubated at 60°C with shaking at 550 rpm for 60 minutes and afterwards were washed three times with appropriate washing buffer. First wash was carried out at 30°C with shaking at 550 rpm for 5 minutes, and subsequent two washes at 20°C. Subsequently, microarray tubes were blocked with freshly prepared blocking solution (containing 2% (w/v) milk powder) at 30°C with shaking at 550 rpm for 15 minutes and diluted streptavidin-conjugated horseradish peroxidase (Poly-HRP Streptavidin) was added to the sample and incubated at 30°C for 15 minutes. This was then followed by a series of three washes at 30°C with shaking at and 550 rpm for 5 minutes. Subsequently, a 3',5,5'-tetramethylbenzidine (TMB) analog (SeramunGrün®, Seramun Diagnostica) was added to each array tube, incubated at the room temperature 10-15 minutes and the array was imaged using a tube reader (Clondiag). Data were analysed using Iconoclust software (Clondiag), which measures the signal intensity and the background for each spot on the array. AMR analyses were performed in duplicate.

2.2.7 Fluorescent *in situ* Hybridization

Fluorescent *in situ* Hybridization **(**FISH) using 16S rRNA oligonucleotide probes was performed accordingly to the previously described method (Daims *et al*., 2005). Specific probes (Table 2.5) for bifidobacteria, lactobacilli/enterococci and *Salmonella* were used to enumerate bacteria of interest. Total bacterial counts were obtained with

nucleic acid stain 4′6-diamidino-2-phenylindole (DAPI). Briefly, faecal slurry samples were collected from batch culture at 0, 5, 10 and 24 hours post inoculation and fixed in ice-cold 4% (w/v) paraformaldehyde for 4-6 hours at 4°C. Subsequently, samples were centrifuged at (16 000 × g) for 5 minutes, washed twice with 0.1M PBS (pH 7.2) and pellet was resuspended in 300 µl PBS/ethanol (50:50). Samples were mixed by vortexing and stored at -20°C until required. For hybridization, samples were diluted in PBS/SDS and a 20 µl aliquot of the diluted sample was pipetted onto Teflon- and poly-L-lysine- coated, six well slides (Tekdon Inc.). Samples were dried at 46°C for 15 minutes and then dehydrated in ethanol using 50%, 80% and 96% (v/v) concentrations (3 minutes in each). Following dehydration samples were again dried at 46°C to allow evaporation of alcohol. For the detection of lactobacilli/enterococci (probe Lab158), a 5 µl of lysozyme was applied to each well and left for 15 minutes at 37°C; washed briefly in water and dehydrated in a alcohol series as described above. A 50 µl mixture containing 5 µl of the probe and 45 µl of the hybridization buffer (HB) was applied onto each well and hybridization was performed for 4 hours using ISO20 oven (Grant Boekel). Following hybridization, slides were transferred into 50 ml wash buffer containing 20 µl DAPI and incubated in a water bath for 15 minutes. Slides were then washed in ice-cold water, dried under compressed air and 5 µl of anti-fade reagent was added to each well. The coverslips were applied and the slides were stored at 4°C until required. Slides were examined under an epifluoresce microscope (Eclipse 400; Nikon, Surrey, United Kingdom).

Bacteria were enumerated according to the following equation:

$$DF \times ACC \times 6732.42 \times 50 \times DF\ sample$$

where:

DF is the dilution factor, ACC is the average cells count of 15 fields of view, 6732.42 number is the area of the well divided by the area of the field of view, 50 is the factor that reverts the cell count to per milliliter of sample, DF sample is the dilution of sample according to the used probe or stain.

Table 2.5 16S rRNA oligonucleotide probes used for bacterial enumeration.

Probe name		**Sequence (5' to 3')**
Short	**Full name**	
Bif164[a]	S-G-Bif-0164-a-A-18	CATCCGGCATTACCACCC
Lab158[b†]	S-G-Lab-0158-a-A-20	GGTATTAGCAYCTGTTTCCA
Sal303[c]	L-S-Sal-1713-a-A-18	AATCACTTCACCTACGTG

[a] Target species: Most *Bifidobacterium* spp. and *Parascardovia dendicolens* (Langendijk *et al*., 1995)

[b] Target species: Most *Lactobacillus*, *Leuconostoc* and *Weissella* spp.; *Lactococcus lactis*; all *Vagococcus*, *Enterococcus*, *Melisococcus*, *Tetragenococcus*, *Catellicoccus*, *Pediococcus* and *Paralactobacillus* spp. (Harmsen *et al*., 1999).

[c] Target species: Different serovars of *Salmonella* spp. (Nordentoft *et al*., 1997).

† Requires lysozyme treatment.

Table adapted from Martin-Pealez *et al*. (2008).

2.3 Mass spectrometry methods

2.3.1 Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF)

Lactobacillus strains were grown on MRS agar as described in section 2.1.1 and fresh overnight culture was used for preparation of samples for MALDI ToF analysis. Method of Mellmann *et al*. (2008) was followed for sample preparation. Briefly, approximately 1 inoculation loop of fresh bacterial culture was resuspended in 300 µl water and 900 µl of ethanol. Samples were centrifuged at 13 000 × g and supernatant was decanted. To extract the cells, 50 µl of 70% formic acid (in water) was added to the bacterial pellet, sample was mixed thoroughly after which 50 µl of acetonitrile was added. Samples were again centrifuged at 13 000 × g for 2 minutes and 1 µl of obtained supernatant was transferred onto wells on a steel MALDI target plate. Supernatants were allowed to dry at room temperature and subsequently overlaid with 1.5 µl saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile-2.5% trifluoroacetic acid mixture (MALDI matrix) and samples were left to dry. Samples were analysed using Bruker Autoflex 2 MALDI-ToF machine (Bruker, Daltonics). The main spectral projection (MSP) spectra were processed with BioTyper software (Bruker, Daltonics) based on a comparison with the BioTyper reference library of MALDI-TOF mass spectra. Sample identification was based on two technical repeats.

2.4 Chromatography methods

2.4.1 High-performance liquid chromatography (HPLC)

SCFA analysis of lactobacilli CFS's and of batch culture samples were performed using an HPLC system and as described previously (Sarbini *et al*., 2011). Briefly, lactobacilli cultures and batch culture samples (section 2.1.14) were centrifuged for 10 minutes at (13 000 × g), supernatants were filter sterilized using 0.22 µm polycarbonate syringe filter and analysed using a HPLC system (LaChrom Merck Hitachi, Poole, Dorset, UK). System was equipped with a pump (L-7100), a refractive index detector (L-7490), and an autosampler (L-7200). For the separation of organic acids the ion-exclusion REZEX ROA-Organic acid column was used (Phenomenex Inc.). During the separation of organic acids the temperature was maintained at 85°C. The eluent used in the system was degassed sulphuric acid diluted in the HPLC-grade H_2O (0.0025 mmol/L); the flow rate was maintained at 0.5 ml/min. Standards for lactic, acetic, propionic, butyric, isobutyric, valeric and isovaleric acids were used at the following concentrations 12.5 mM, 25 mM, 50 mM, 75 mM, and 100 mM. Quantification of the samples was gained through calibration of the standards curves and results were the average from three replicates.

2.5 *In vitro* methods

2.5.1 Porcine intestinal epithelial cell line (IPEC-J2)

2.5.1.1 IPEC-J2 monolayers

IPEC-J2 cell line (derived from jejunum of an un-suckled 1-day old piglet) when used as monolayers were maintained according to Schierack *et al*. (2006). All reagents unless indicated otherwise were purchased from Sigma-Aldrich. Briefly, cells were cultured in a mixture of DMEM/Ham's F12 (1:1) medium supplemented with 5% (v/v) foetal calf serum (FCS), ITS media supplement (5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite), 5 ng/ml epidermal growth factor, 1% (v/v) non-essential amino acids, 1% (v/v) L-glutamine and gentamycin (50 µg/ml), hereafter referred as IPEC-J2 medium. Cells were maintained in a 5% CO_2 atmosphere.

For experiments cells were seeded in 24-well plates (Nunc) at a density of 2×10^5 cells per well and grown to confluent monolayers. During the assays cells were maintained in IPEC-J2 depleted of antibiotics.

2.5.1.2 Three-dimensional IPEC-J2 model

The porcine jejunal 3D cell culture model was based upon the previous methods, (Nickerson *et al*., 2001; Searle *et al*., 2010) and Dr J. W. Collins personal communication. For the 3D cell model standard IPEC-J2 medium was additionally supplemented with glucose, galactose and fructose (1 g/l, 0.25 g/l, 0.13 g/l) (Goodwin *et al*., 1993). IPEC-J2 monolayers were cultured as described above, in tissue culture flask. Cells were washed with Hank's balanced salt solution (HBSS) and detached using 0.25% trypsin for 5 minutes. Subsequently, fresh medium was added to remove trypsin, cells were centrifuged and then resuspended in a complete fresh IPEC-J2-3D medium to yield 2×10^5 cells/ml. Cell suspensions were mixed with 5mg/ml Cytodex™ microcarrier beads (Cytodex-3, size 133-225 µm) in a 50 ml Corning® centrifuge tube. Before use microcarrier beads were hydrated in 0.1M PBS (pH 7.2) and autoclaved according to supplier instructions. In order to facilitate the initial cell-bead attachment, suspensions were incubated at 37°C, 5% CO_2 for approximately 30 minutes. Subsequently, cell suspensions were introduced to the 50 ml rotating wall vessel (RWV) (Synthecon), air bubbles removed and cultured at a rotation starting at 13.7 rpm to maintain the cell-bead aggregates in the suspension. Cells were incubated for two days without media change to allow further attachment of cells. Circa 80% of the medium was replenished every second day and rotation was gradually increased over time as necessary. Cells were cultured in RWV (37°C, 5% CO_2) for 20-22 days. Following this time (day of the assay) cells were removed from the RWV, resuspended in IPEC-J medium (without antibiotics) to yield 5×10^5 cells/ml of suspension. To obtain the cell number in 1 ml, the cell aggregate suspensions were trypsinized and passed through a 70 µm sieve in order to separate them from the microcarrier beads. Following separation from the microcarrier bead fresh IPEC-J2 medium was added and cells were counted using a haemocytometer. For experiments cell aggregates were seeded into 1.5 ml centrifuge tubes.

2.5.2 *L. plantarum* B2028 and lactulose adherence assay.

Adhesion assay was performed as previously described (Dibb-Fuller *et al*., 1999) with slight modifications. Briefly, *L. plantarum* B2028 inocula were prepared by centrifugation (2447 × g) for 10 minutes, the supernatant was decanted, the pellet washed in 0.1M PBS (pH 7.2) and resuspended in IPEC-J2 medium to yield 5×10^7 cfu/ml. Cell-free supernatants were prepared by filter sterilization as described in

section 2.1.8. Cells were washed twice with Hank's balanced salt solution (HBSS) and 1 ml of the bacterial inoculum containing accordingly: *L. plantarum* B2028, *L. plantarum* B2028 + 1% (w/v) LAC, both cultured in standard MRS and *L. plantarum* B2028 cultured in MRSL (with 1% (w/v) LAC) was added to a 24-well plate. Wells containing IPEC-J2 medium only were included as a control conditions. Plates were incubated at 37°C and in the presence of 5% CO_2 for up to 2 hours. Subsequently, cells were washed three times with HBSS, disrupted with 1% Triton X-100 (Sigma-Aldrich) and mechanical stirring. Serially diluted cell suspensions ($10^0 – 10^6$) were plated onto MRS agar and incubated microaerophilically at 37°C for 24 hours.

2.5.3 *S.* Typhimurium viability and inhibition of invasion assay

To evaluate the effect of pre-treatment with *L. plantarum* B2028 supernatant on *S.* Typhimurium SL1344nal^r viability and invasion, a previously described method was followed (Coconnier *et al.*, 1997; Makras *et al.*, 2006). Briefly, *S.* Typhimurium SL1344nal^r cells (10^8 cfu/ml) were co-incubated with IPEC-J2 medium containing 10% (v/v) of *L. plantarum* B2028 cell-free CFS's (CFS/CFSL), pH control medium (MRS/MRSL) and lactic acid control medium (MRS-LA/MRSL-LA) at pH 3.8 and prepared as described previously in section 2.1.8. *S.* Typhimurium SL1344nal^r cells incubated in IPEC-J2 medium only and medium without bacterial inoculum added were included as controls. Following 1 hour incubation at 37°C serially diluted cell suspensions ($10^0 – 10^7$) were plated onto LB-G agar for enumeration. Subsequently, *S.* Typhimurium SL1344nal^r cells were washed in 0.1M PBS (pH 7.2) and resuspended in IPEC-J2 medium to yield 5×10^7 cfu/ml and the invasion assay was conducted as described below for competition assays (section 2.5.4).

2.5.4 Competition assays using IPEC-J2 monolayers and 3D model

Competition assays were performed as previously described (Dibb-Fuller *et al.*, 1999; Dibb-Fuller *et al.*, 2001; Mappley *et al.*, 2011; Searle *et al.*, 2009; Searle *et al.*, 2010). Briefly, *L. plantarum* B2028 and *S.* Typhimurium SL1344nal^r culture (section 2.1.1) was centrifuged (2447 × g) for 10 minutes and the supernatant was decanted. Bacterial pellet was washed in 0.1M PBS (pH 7.2) and subsequently resuspended in IPEC-J2 medium to yield 10^8 cfu/ml. Subsequently, bacterial inocula (Table 2.6) were added to 24-well plates (Nunc) (for monolayers) or 1.5 ml centrifuge tubes (for 3D cell aggregates) to a final concentration of 5×10^7 cfu in a well/tube in a 1 ml volume.

Wells/ centrifuge tubes containing IPEC-J2 medium only were included as a control conditions. Inoculated plates, one for adhesion and one for invasion and centrifuge tubes were incubated at 37°C (in the presence of 5% CO_2) for 60 minutes.

Following incubation, to count the *S.* Typhimurium SL1344nal^r associated, the cells were washed three times with HBSS and disrupted with 1% Triton X-100 (Sigma-Aldrich) using magnetic stirrer. To distinguish the number of *S.* Typhimurium SL1344nal^r invaded, the cells were washed twice with HBSS and inoculated with 1 ml of media containing 100 µg/ml gentamycin solution (Sigma-Aldrich) and incubated for additional 2 hours. Subsequently, cells were washed three times with HBSS and disrupted as described above. To determine the number of associated and invaded *S.* Typhimurium SL1344nal^r, 10-fold serial dilutions ($10^0 – 10^{-4}$) were plated onto LB-G agar (Oxoid). The number of adhered *S.* Typhimurium SL1344nal^r was obtained by subtracting the number of invaded bacteria from the number of associated bacteria. All assays were conducted in duplicate on three separate occasions.

Table 2.6 Experimental conditions used in the competition assays using IPEC-J2 cell line (monolayers and 3D cells) and porcine *in vitro* organ culture (IVOC) model.

Condition	Experimental strategy
SL	*S.* Typhimurium SL1344nal^r
MRSL pH 3.8	*S.* Typhimurium SL1344nal^r + 10% (v/v) MRSL[a] pH 3.8
LAC	*S.* Typhimurium SL1344nal^r + 1% (w/v) LAC
Lp	*S.* Typhimurium SL1344nal^r + *L. plantarum* B2028
Lp + LAC	*S.* Typhimurium SL1344nal^r + *L. plantarum* B2028 + 1% (w/v) LAC
Lp + CFSL	*S.* Typhimurium SL1344nal^r + *L. plantarum* B2028+ 10% (v/v) CFSL[b]
CFSL	*S.* Typhimurium SL1344nal^r + 10% (v/v) CFSL[b]

[a] MRS broth with 1% (w/v) lactulose as a main carbon source.

[b] Cell free supernatant of *L. plantarum* B2028 cultured in MRSL broth.

2.5.5 Giemsa assay

IPEC-J2 monolayers were used and maintained as described in section 2.5.1.1 with a slight modification as cells were grown in 24-well plates on 13 mm cover slips. Each well was inoculated with inocula containing *L. plantarum* B2028 cells, cells with 10% (v/v) CFS, or with 1% (w/v) LAC. Media only and media containing 10% (v/v) CFS and 1% (w/v) LAC were also added but depleted of bacterial cells. Plates were

incubated for 60 min as described for adhesion assays. Cells were stained with 10% Giemsa stain (Sigma-Aldrich) for 60 minutes and subsequently washed three times with distilled water and differentiated with 1% (v/v) acetic acid for 2 minutes followed by a wash with sterile distilled water. Cover slips containing cells were then removed from the 24-well plate and mounted on glass slides using DPX mountant (mixture of distyrene, a plasticizer and xylene) (Sigma-Aldrich). Slides were examined under oil immersion using light microscope (Olympus CX21, × 1000). Assays were performed in duplicate on two separate occasions.

2.5.6 Porcine *in vitro* organ culture

In vitro organ culture association assays were performed as previously described (Collins *et al*., 2010). Briefly, four 6-week old male commercial pigs were housed and fed a commercial un-medicated diet and water *ad libitum* for 7 days prior to the study. Pigs were euthanased by stunning and exanguination and subsequently placed on their dorsal recumbency and a mid-line incision was made. At *post-mortem* the whole intestinal tract was exteriorized and the jejunum and spiral colon located and sampled aseptically. Tissue samples were immediately placed in pre-cooled IVOC medium and transported to the laboratory on ice. The IVOC medium consisted of: complete RPMI 1640 medium containing 10% (v/v) fetal calf serum (FCS), 0.25% lactalbumin hydrosylate, 75 mM mercaptoethanol, 0.2 µg/ml hydrocortisone (1:1 chloroform/ethanol), 0.1 µg/ml insulin, 2 mM L-glutamine and L-aspartate (Collins *et al*., 2010; Girard *et al*., 2005). All reagents unless indicated otherwise were purchased from Sigma-Aldrich. Tissues were washed in IVOC medium, aseptically trimmed into *circa* 2cm x 2cm squares and immobilized in CellCrowns™ (Scaffdex) to obtain fixed mucosal surface of the tissue explants. Subsequently, immobilized intestinal explants from jejunum and colon were placed into pre-filled with IVOC medium (500 µl) 24-well plates (Nunc) with the mucosal surface facing upwards.

For association assays, *L. plantarum* B2028 and *S.* Typhimurium SL1344nal^{r} bacterial inocula were prepared and administered essentially as described for the competition assays (Table 2.6) with the exception that the final bacterial concentration in a well (1ml volume) was 10^{8} cfu. Plates were incubated at 37°C (in the presence of 5% CO_2) for 30 minutes with gentle agitation. Following the incubation tissue samples were washed in and placed in 9 ml 0.1M PBS (pH 7.2) and homogenized for bacteriological analysis. Serial dilutions (10^{0} – 10^{-6}) were plated out onto BGA

supplemented with 15 µg/ml nalidixic acid. Uninfected tissues were incubated with media only as controls. Assays were conducted in quadruplicate on two separate occasions.

2.6 *In vivo* methods

2.6.1 Animals and management

A total of twenty four, cross breed, mixed sex pig with a mean initial weight of 7.98 ± 0.7 kg were used for the study. Animals were weaned at 4 weeks of age, faecal samples were collected from sows (n = 3) and piglets and tested for the presence of *Salmonella* before the trial commencement. Pigs were randomly divided into four equal groups of six on the basis of their mean body weight and housed in a bio-containment facility (CLII). Each pen was equipped with a feeder and water supply from a water tray and from a nipple. Pens, feeders and water trays were cleaned on a daily basis. Pigs were fed commercial un-medicated pelleted feed (Lillico Attlee, Wm. Lillico & Son Ltd), according to their daily requirements (ASU Unit, AHVLA) and water was provided *ad libitum*. The left over feed was weighed every morning. Biosecurity measures were implemented in order to avoid cross contamination. These studies were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and were approved by the AHVLA ethics committee. All procedures were carried out at the AHVLA Coomblands Farm.

2.6.2 Bacterial strains preparation

S. Typhimurium SL1344 nal^r and *L. plantarum* B2028 were cultured as previously described in LB-G and MRS broth respectively, centrifuged (2447 × g) for 10 minutes and the supernatant was decanted. For *S*. Typhimurium SL1344 nal^r the pellet was and resuspended in 0.1M PBS (pH 7.2) to yield 10^9 cfu/ml bacteria and further diluted in sterile water to yield 5×10^7 cfu/ml. *L. plantrarum* B2028 was resuspended in sterile water and mixed with the small portion of feed for each pig to receive 10^{10} cfu/pig/day.

2.6.3 Experimental design and diets

Once distributed into four separate pens, the animals were allowed 7 days of acclimatisation after which the experimental diets were provided. The experimental treatment consisted of pelleted control diet without additives (Control), diet containing

1% (w/w) (15 ml/kg) lactulose given as a Duphalac syrup (LAC), diet containing 10^{10} cfu of *L. plantarum* B2028/animal/day (Lp) and diet containing 10^{10} cfu of *L. plantarum* B2028/animal/day together with 1% (w/v) (15 ml/kg) lactulose (Lp + LAC). Once stabilised on the above diets (6 days), each piglet in the all four groups was challenged with *S.* Typhimurium SL1344nal^r (5×10^8 cfu in 10 ml sterile water) administered by oral gavage (day 0). Approximately 30 minutes before the challenge animals were orally dosed with 10% (w/v) sodium bicarbonate solution to neutralise stomach acid (20 ml). Clinical performance was monitored throughout the entire study and samples were collected as detailed in section 2.6.4. At the end of the study, 10 days after *Salmonella* challenge all animals were humanely sacrificed for *post-mortem* examination.

2.6.4 Experimental monitoring and animals handling

All animals were examined clinically and scored as per Table 2.7 throughout the duration of the study. Feed consumption was recorded daily and body weights were taken weekly. Rectal temperature and faecal samples were collected from each piglet by rectal digital insertion on three separate occasions before *Salmonella* challenge, while animals were fed the experimental diet.

Table 2.7 Clinical score evaluation.

Clinical score	Diarrhoea	Feed consumption	Behaviour	Temperature
0	None	Normal	Active	Normal (38.5 – 40°C)
1+	Slight	Slightly off food	Active if stimulated	Raised (40.5 – 41°C)
2+	Watery (yellow, watery, +/- blood)	Off food	Inactive, when stimulated	Fever (above 41°C)

Following *S.* Typhimurium SL1344nal^r challenge, sampling was performed on a daily basis. Faecal samples (approx. 5 g) from each pen were collected from the floor daily and pooled for *Salmonella* testing.

2.6.5 Bacterial isolation and enumeration

Direct isolation and enumeration of *S.* Typhimurium SL1344nal^{r} from faeces were obtained by resuspending 10% (w/v) freshly collected samples in a buffered peptone water (BPW) (Edel and Kampelmacher, 1973), plating out serially diluted samples (10^{0}-10^{-5}) onto BGA supplemented with nalidixic acid 15 µg/ml and incubating at 37°C for 16 hours (Searle *et al*., 2009). For initial enrichment, resuspended in BPW samples were incubated at 37°C for 18 ± 2 hours. Subsequently, 100 µl of the overnight incubated broth was inoculated onto modified semisolid Rappaport-Vassiliadis medium (MSRV) (Aspinall *et al*., 1992; De Smedt *et al*., 1986) as a three equal drops. MSRV plates were incubated at 41.5°C for 24 hours. Plates which were negative for growth were allowed further 24 hours incubation and examined for growth. Subsequently, a 1 µl loop was used to pick the material from the edge of the definite growth, which was streaked onto Rambach agar and BGA agar supplemented with 15µg/ ml of nalidixic acid and colonies were allowed to develop for 24 hours at 37°C.

Tissues collected at *post-mortem*, were weighed (1 g where possible), homogenised in BPW (10% w/v) and serially diluted samples (10^{0}-10^{-5}) were plated onto BGA supplemented with 15µg/ ml of nalidixic acid. Samples were enriched as described above and a 100 µl aliquot was taken from the samples that were negative upon direct plating.

Lactobacilli numbers were determined by diluting fresh faecal samples 10% (w/v) in BPW and 10-fold serial dilutions in 0.1M PBS (pH 7.2) (10^{-1}-10^{-8}) were prepared and plated out on MRS agar (De Man *et al*., 1960). Plates were incubated at 37°C microaerophilically in GasPak jars using a GasPak™ plus system (BBL™) and colonies were allowed to develop for 48 hours. At four distinct time points during the study, feacal samples were subjected to PCR analysis to test for *L. plantarum* spp. presence (Kwon *et al*., 2004).

For *E. coli* and coliform enumeration of faeces, samples were prepared as above and plated onto a Chromagar ™ ECC (Randall *et al*., 2009). Plates were incubated at 37°C for 24 hours and all blue *E. coli* and mauve coliforms were counted.

2.6.6 *Post-mortem* examination

Ten days following pathogen challenge all pigs were euthanased by stunning and exanguination and subsequently placed in dorsal recumbency for a mid-line incision to

be made. Faecal samples were collected from rectum from all experimental animals. Tissues (jejunum, ileum, colon, caecum, rectum and mesenteric lymph nodes) from three pigs from each experimental group were sampled at *post-mortem* for bacteriology and histopathology.

2.7 Statistical analysis

Statistical analysis of the data presented within this thesis was performed using GraphPad Prism® version 5 program (GraphPad Software, LaJolla, CA, USA). Unless otherwise indicated the evaluation of statistically significant differences between results from the treatment groups and the treatment groups and the control were determined by repeated-measures One-way analysis of variance (ANOVA) and Bonferroni post test. For the studies where two factors (time and treatment) were taken into account, the repeated-measures Two-way ANOVA was used followed by the Bonferroni post test. The area under the curve (AUC) was calculated by the trapezoidal method with GraphPad Prism® version 5 program. The mean AUC values were compared using One-way ANOVA and Bonferroni post test. For analysis of *Salmonella* counts from the *in vivo* trial, the scoring system was applied and data between treated and control group compared.

Statistical significance was accepted at $P<0.05$ and differences among means with $0.05<P<0.10$ were accepted as tendencies to differences. All data within this thesis are presented as the mean with the standard error of the mean (SEM). The significance (*P* value) was visualized on graphs using an asterisk scale system (Table 2.8).

Table 2.8 *P* value summary.

***P* value**	**Symbol**
≤ 0.001	***
> 0.001 to ≤ 0.01	**
> 0.01 to ≤ 0.05	*
> 0.05	ns

Chapter 3

Salmonella and *Lactobacillus* isolate characterisation towards selection of an effective synbiotic that reduces *S.* Typhimurium colonisation

3.1 Introduction

The control of *Salmonella* colonisation in animals is of primary importance in attempts to reduce the incidence of human salmonellosis. As a consequence of increasing antibiotic resistance in zoonotic pathogens, the use of antimicrobial growth promoters that were associated with pathogen control and improvement in zootechnical parameters was banned in EU in 2006 (Cogliani *et al*., 2011).

A number of studies have indicated that probiotics may be used as a control strategy against common intestinal pathogens in livestock (Brashears *et al*., 2003; Casey *et al*., 2007) and genera including lactobacilli and bifidobacteria are commonly used (Saarela *et al*., 2000). Nevertheless, there remains a continual challenge to find suitable probiotic candidates that replace and/or supplement antibiotic use (Verstegen and Williams, 2002). The concept of using one organism to exclude another is not recent, the protective role of native gut microbiota in limiting the colonisation of *Salmonella* in chicks was demonstrated nearly 40 years ago (Nurmi and Rantala, 1973). In addition to probiotics another widely used approach is to employ prebiotics in animal nutrition, or the combination of prebiotics and probiotics, termed synbiotics. Prebiotics are non-digestible, complex and variable chained length carbohydrates that are often both synthesised and catabolised by probiotic strains. Therefore, a diet supplemented by these compounds (FOS, GOS, MOS, XOS etc.) will see these carbohydrates arrive in the distal part of the alimentary tract where they are catabolised by organisms that have the capability to do so. Given synthesis of prebiotics is by probiotic strains, these will be selectively enriched as they possess the relevant

enzymic capabilities. Consequently beneficial microbiota will be enhanced and increased resistance to enteric pathogens is provided (Patterson and Burkholder, 2003).

The importance of accurate probiotic strain identification and characterisation for its selection and industrial suitability is evident and has been pointed out by numerous researchers (Klaenhammer and Kullen, 1999; Saarela *et al*., 2000; Temmerman, 2004). Therefore, within the first section of this chapter it was deemed prudent to characterise and confirm the identity of the organisms to be used in this study; *Salmonella* as the target pathogen for control and the lactobacilli strains as potential probiotics. The ultimate goal of this work however will be to construct an effective synbiotic combination that reduces colonisation of *S.* Typhimurium in pigs. Although, the mechanism of probiotic action has not yet been fully elucidated, it has been documented that probiotic strains are able to produce different antimicrobial substances e.g. organic acids, hydrogen peroxide, carbon dioxide and bacteriocins (Castillo *et al*., 2011; Fayol-Messaoudi *et al*., 2005; Servin, 2004). It has been suggested that the major fermentation product of lactic acid bacteria, lactic acid, is a significant component of their antimicrobial activity *in vitro* (Makras *et al*., 2006). In addition to the pH lowering effect of lactic acid Alakomi *et al*. (2000) demonstrated its role as an outer membrane disrupting agent of Gram-negative bacteria such as *S.* Typhimurium, *E. coli* and *P. aeruginosa*.

The phenotypic and molecular characterisation of *Lactobacillus* isolated used in this study is demonstrated in this chapter. Furthermore, in order to aid in the selection of prospective probiotics, initial screening to evaluate the antimicrobial activity towards *S.* Typhimurium SL1344nal^{r} by lactobacilli is demonstrated. This chapter also discusses the evaluation of a candidate prebiotic inclusion on the fermentative activity of porcine microbiota including the candidate probiotic.

3.2 Results

3.2.1 General characterisation of *Salmonella* isolates

To reconfirm *S.* Typhimurium isolates identity standard bacteriological testing was performed as described in sections 2.1.2 and 2.1.3. Gram staining confirmed the isolates to be Gram negative (red-pink) rod shaped bacteria. All isolates were catalase positive and oxidase negative. The isolates were tested for their biochemical profiles using API20E strips as described in section 2.1.4 and the results were analysed using Apiweb™. The biochemical profiles were representative for *Salmonella* spp. for all isolates with the high identification profiles (99.9%). All isolates were able to ferment D-glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, D-melibiose, L- arabionose. All isolates produced hydrogen sulphide (H_2S) gas. Finally, having confirmed that all isolates were *Salmonella*, their serovar designation was also confirmed by slide agglutination test with each isolate was confirmed to be Typhimurium.

For all future studies however, a nalidixic acid resistant derivative of *S.* Typhimurium SL1344 was selected in order to ease the recovery from the complex experimental environments. The resistance to nalidixic acid was confirmed by plating out on BGA containing 15 µg/ml nalidixic acid and this medium was used for the pathogen recovery.

3.2.2 Phenotypic and molecular characterisation of lactobacilli isolates

For presumptive lactobacilli, obtained from the AHVLA culture collection, the Gram staining confirmed the isolates to be Gram positive (purple) rod shaped bacilli that lacked spores. All isolates were catalase and oxidase negative. *Lactobacillus* strains characterised using API50 CHL strips allowed differentiation between species on the basis of their typical fermentation profiles as described in section 2.1.4. The API identification for each isolate gave a range of probability as follows (given from the highest): *L. plantarum* B2028, B2989, B2994 and B2996 99.90%; *L. rhamnosus* B2988, B2998, B2987 99.70-96.50%; *L. bulgaricus* B2999 (*L. delbrueckii* subspecies) was identified as *L. delbrueckii* 88.60%; *L. acidophilus* B2993, B2990 76.40 & 47%; *L. fermentum* B2992 59.80%; *L. bulgaricus* B2991, B2997 57.80% & 45.5%. Moreover, *L. reuteri* B2026 was identified by API kit as *L. fermentum* 99.30%; *L. casei* B2986 & B2995 was identified as *L. paracasei* 80% (Table 3.1).

In addition, isolates were identified at the species level using multiplex PCR,

sequencing of the 16S rDNA gene and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) profiling as described in section 2.3.1. The multiplex PCR was performed using the primers derived from the integrated sequences of 16S rRNA and 23S rRNA genes that allow identification of seven *Lactobacillus* species (Kwon *et al*., 2004). All isolates with the exception of *L. fermentum* B2992 and *L. buchneri* B2997 were identified accordingly to a species level (Table 3.1). In addition, the established PCR (Marchesi *et al*., 1998) using universal primers 63f and 1387r has been used to amplify 16S rRNA gene of each isolate and the amplicons were sequenced (see section 2.2.5). Forward and reverse sequences were aligned and compared on the GenBank database. Species matches and the accession number are shown in a Table 3.1. Finally, MALDI-TOF MS profiles compared with database profile gave accurate ID to *L. plantarum, L. fermentum, L. buchneri, L. acidophilus* (one isolate). Again, *L. bulgaricus* was identified as *L. delbrueckii*. Moreover, *L. casei* was identified as *L. paracasei* and one of *L. rhamnosus* strains was identified in the first repeat as *L. casei* and when profiling was repeated as *L. zeae*. Similarly, in the instance of one of *L. acidophilus* strains it was recognised to be *L. ultunensis* or *L. helveticus*. These data are summarized in Table 3.1.

Table 3.1 The origin of the *Lactobacillus* strains and their phenotypic and molecular identification. Species identification match using API 50CH, MALDI-TOF MS, Multiplex PCR and 16S rRNA sequencing.

Strain bead number	Species as defined by original source definition	Origin	API 50CH ID		MALDI-TOF MS	Multiplex PCR[a]	16S rRNA sequencing		
			Species	ID %	Species	Species	Match species	Match accession	ID %
B2028 (JC1)	*L. plantarum*	Swine	*L. plantarum*	99.90%	*L. plantarum*	*L. plantarum*	*L. plantarum*	JQ278711.1	100%
B2026	*L. reuteri*	Swine	*L. fermentum*	99.30%	*L. reuteri*	*L. reuteri*	*L. reuteri*	JN981867.1	98%
B2986	*L casei*	Human	*L. paracasei*	80.00 %	*L. paracasei*	*L. casei*	*L. casei*	JN560879.1	99%
B2987	*L. rhamnosus*	Human	*L. rhamnosus*	96.50%	*L. rhamnosus*	*L. rhamnosus*	*L. rhamnosus*	HQ293051.1	97%
B2988	*L. rhamnosus*	Human	*L. rhamnosus*	99.70%	-	*L. rhamnosus*	*L. rhamnosus*	JQ621982.1	98%
B2989	*L. plantarum*	Vegetable	*L. plantarum*	99.90%	*L. plantarum*	*L. plantarum*	*L. plantarum*	GU451062.1	98%
B2990	*L. acidophilus*	Human	*L. acidophilus*	47.00%	*L. ultunensis/helveticus*	*L. acidophilus*	*L. acidophilus*	EU878007.1	99%
B2991	*L. bulgaricus*	Dairy	*L. bulgaricus*	57.80%	*L. delbrueckii*	*L. delbrueckii*	*L. bulgaricus*	FJ878007.1	98%
B2992	*L. fermentum*	Vegetable	*L. fermentum*	59.80%	*L. fermentum*	-	*L. fermentum*	EU626018.1	99%
B2993	*L. acidophilus*	Human	*L. acidophilus*	76.40%	*L. acidophilus*	*L. acidophilus*	*L. acidophilus*	JQ350808.1	100%
B2994	*L. plantarum*	Vegetable	*L. plantarum*	99.90%	*L. plantarum*	*L. plantarum*	*L. plantarum*	GU451062.1	98%
B2995	*L. casei*	Dairy	*L. paracasei*	80.00%	*L. paracasei*	*L. casei*	*L. casei*	JN560917.1	100%
B2996	*L. plantarum*	Vegetable	*L. plantarum*	99.90%	*L. plantarum*	*L. plantarum*	*L. plantarum*	AM279764.2	100%
B2997	*L. buchneri*	Vegetable	*L. buchneri*	45.50%	*L. buchneri*	-	*L. buchneri*	AB425940.1	100%
B2998	*L. rhamnosus*	Dairy	*L. rhamnosus*	99.60%	*L. casei/zeae*	*L. rhamnosus*	*L. rhamnosus*	HQ293051.1	100%
B2999	*L. bulgaricus*	Dairy	*L. delbrueckii*	88.60%	*L. delbrueckii*	*L. delbrueckii*	*L. bulgaricus*	EU547306.1	100%

[a] Multiplex PCR according to Kwon *et al*. (2004) detects *L. acidophilus*, *L. casei*, *L. delbrueckii*, *L. gasseri*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*; -, result inconclusive;

3.2.3 Antibacterial activity of lactobacilli against *S*. Typhimurium

3.2.3.1 Colony overlay assay

Antimicrobial activity of 16 lactobacilli isolates (Table 3.1) against *S.* Typhimurium SL1344nal^r was investigated using a colony overlay assay following the method of Barbosa *et al*. (2005) and as described in section 2.1.7. All *Lactobacillus* isolates generated a zone of inhibition against *S.* Typhimurium SL1344nal^r (hereinafter referred to as STm SL1344nal^r) (Table 3.2). The strongest inhibition was demonstrated by *L. plantarum* B2028, *L. plantarum* B2989 and *L. acidophilus* B2990 with the mean inhibition zones measuring 19.66 ± 1.01 mm, 17.53 ± 1.08 mm and 17.15 ± 0.68 mm in diameter respectively. The least potent with the inhibition zones measuring 7.85 ± 0.75 mm, 9.69 ± 2.44 mm, 5.11 ± 1.05 mm, 8.64 ± 1.29 mm in diameter were *L. reuteri* B2026, *L. fermentum* B2992, *L. buchneri* B2997, *L. bulgaricus* B2999. These data suggest diffusible substrates produced by each *Lactobacillus* isolate tested are likely to be causing inhibition of STm SL1344nal^r.

3.2.3.2 Characterisation of cell free supernatants from lactobacilli: pH and organic acid production

A conditioned medium assay where lactobacilli cell free supernatants (CFSs) were included at 10% dilution factor into LB-G broth inoculated with STm SL1344nal^r was performed as described in section 2.1.8.

Twelve of the 16 CFSs tested inhibited the growth of STm SL1344nal^r significantly as defined by AUC calculations (Table 3.2). CFS from isolates B2026, B2993, B2997 and B2999 failed to reduce growth, whilst twelve gave a significant reduction of *Salmonella* growth. Isolates B2989 (*L. plantarum*), B2028 (*L. plantarum*) and B2990 (*L. acidophilus*) exerted greatest inhibition of growth with AUC values of 1.35 ± 0.69, 1.83 ± 0.63 and 2.74 ± 0.28 respectively ($P<0.001$). Interestingly, when CFS of *L. fermentum* B2992 was used the growth of STm SL1344nal^r was slower, but over time the final OD was similar to a final OD of control used. At the final reading point (24 hours) STm SL1344nal^r OD was significantly different from the control in the presence of 11 CSFs tested (Figure 3.1). These findings were comparable with the results obtained previously from the colony overlay assay.

Lactic acid bacteria (LAB) whether hetero- or homo-fermentative are known to produce organic acids, notably lactic acid, as metabolic end products and low pH is

known to inhibit the growth of many Enterobacteriaceae including *S.* Typhimurium. Thus, the CFS from each isolate was examined. The pH of the supernatants ranged from pH = 3.75 ± 0.01 for *L. plantarum* B2028 and B2989 to pH = 5.35 ± 0.10 for *L. bulgaricus* B2991 (Table 3.2). HPLC analyses were performed to quantify the production of organic acids in CFS. The amount of lactic acid ranged from 47.8 mM for *L. bulgaricus* B2999 to 145 mM for *L. plantarum* B2028. In general, it was observed that the greater the inhibition of STm SL1344nal^r the lower the pH of the supernatant and the higher concentration of lactic acid (above 100 mM) (Table 3.2).

3.2.3.3 Hydrogen peroxide production

The ability of lactobacilli strains to produce H_2O_2 was evaluated as described previously in section 2.1.6. Of the sixteen *Lactobacillus* isolates only *L. acidophilus* B2990, *L. acidophilus* B2993, *L. bulgaricus* B991 and *L. bulgaricus* B2999 were H_2O_2 producers.

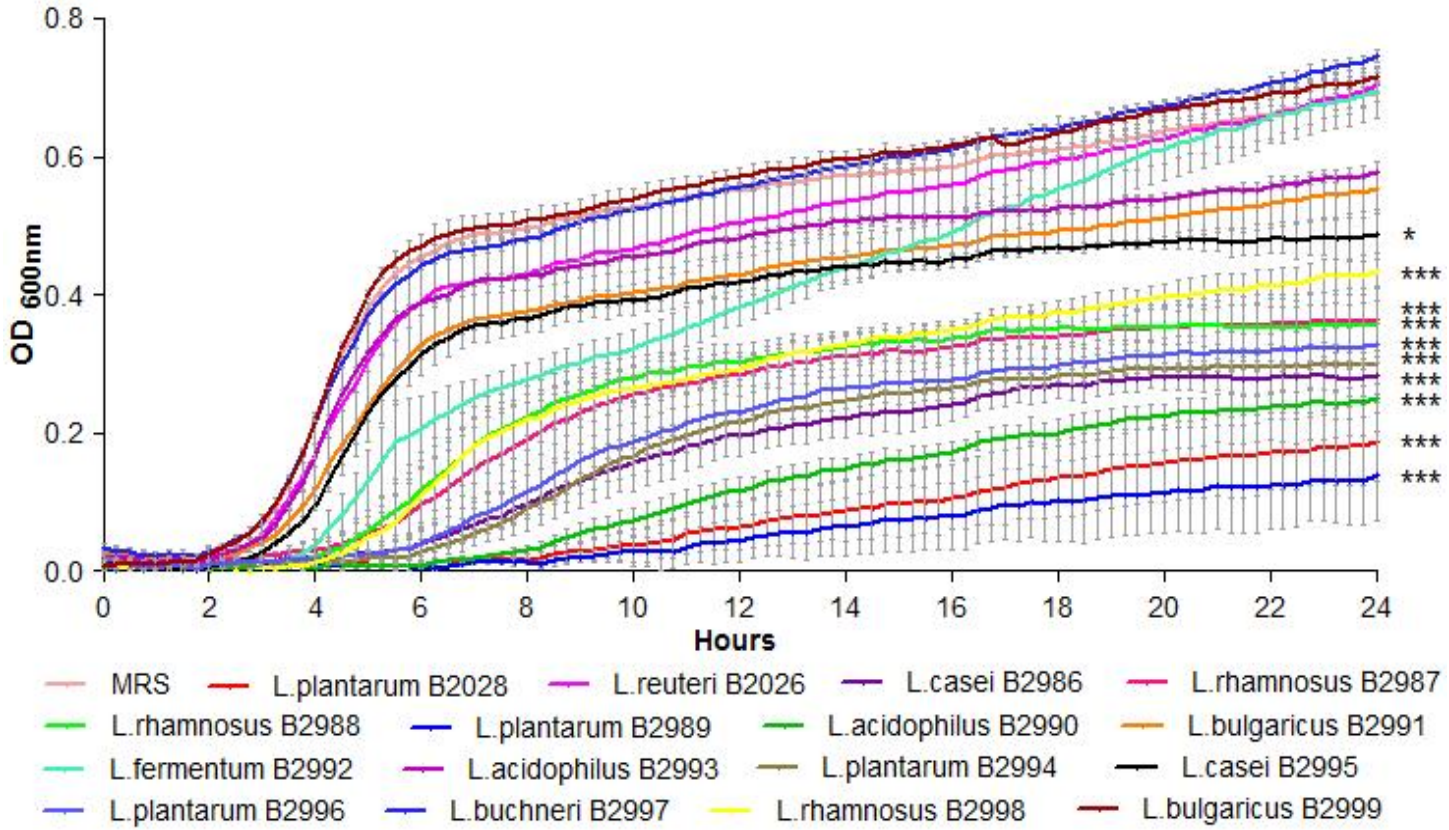


Figure 3.1 Growth of STm SL1344nal^{r} in LB-G broth supplemented with 10% (v/v) non pH adjusted cell-free supernatants of *Lactobacillus* isolates. The CFSs are denoted by their AHVLA reference number. MRS broth (pH 5.8) was added to *Salmonella* culture as a control. Cultures were incubated at 37°C for 24 hours and the OD was measured at 600nm (OD_{600nm}). The assay was performed in triplicate on three separate occasions and the SEM is shown. Significantly different from the control OD values at the final reading point are indicated by * $P<0.05$ and *** $P<0.001$.

Table 3.2 The inhibitory effect of lactobacilli supernatants on STm SL1344nal^{r}, production of hydrogen peroxide and organic acids by lactobacilli. MRS broth (pH 5.8) was included as a control. Assays were conducted in triplicate and SEM is shown.

Isolate	Hydrogen peroxide[a]	Colony overlay assay inhibition zone (mm)[b]	10% CFS growth assay (Mean AUC)[c]	CFS[d] pH	CFS[d] lactic and SCFA concentration (mM)			
					Lactic	Acetic	Butyric	Propionic
L. plantarum (B2028)	-	19.66 ± 1.01(+++)	1.83 ± 0.63 ***	3.75 ± 0.01	145 ± 8.95	97.6 ± 0.20	35.3 ± 4.37	6.7 ± 1.14
L. reuteri (B2026)	-	7.85 ± 0.75(+)	10.67 ± 0.93 ^{NS}	4.43 ± 0.17	50.8 ± 3.65	105 ± 15.1	18.8 ± 0.58	2.7 ± 2.19
L. casei (B2986)	-	15.46 ± 0.63(++)	3.95 ± 0.52 ***	3.84 ± 0.03	129 ± 7.04	106 ± 3.44	20.2 ± 0.02	3.0 ± 1.19
L. rhamnosus (B2987)	-	15.54 ± 1.19(++)	5.54 ± 0.75 ***	3.82 ± 0.01	116 ± 9.91	103 ± 12.8	24.7 ± 0.35	6.4 ± 1.22
L. rhamnosus (B2988)	-	15.01 ± 0.46(++)	5.74 ± 0.61 ***	3.82 ± 0.02	115 ± 2.67	97.7 ± 4.02	20.8 ± 0	2.9 ± 2.14
L. plantarum (B2989)	-	17.53 ± 1.08(+++)	1.35 ± 0.69 ***	3.75 ± 0.01	139 ± 9.09	98.7 ± 1.45	30.3 ± 0.68	2.9 ± 0.69
L. acidophilus (B2990)	+	17.15 ± 0.68(+++)	2.74 ± 0.28 ***	3.81 ± 0.05	133 ± 4.21	92.6 ± 5.51	19.9 ± 0.65	7.7 ± 1.52
L. bulgaricus (B2991)	+	12.97 ± 0.30(++)	8.88 ± 0.69 *	5.35 ± 0.10	95.7 ± 10.2	116 ± 14.1	21.4 ± 0.40	3.7 ± 2.08
L. fermentum (B2992)	-	9.69 ± 2.44(+)	8.69 ± 0.66 *	4.13 ± 0.01	103 ± 3.29	116 ± 0.79	24.3 ± 1.76	4.4 ± 0.08
L. acidophilus (B2993)	+	13.50 ± 0.76(++)	9.79 ± 0.05 ^{NS}	4.07 ± 0.18	76.4 ±11.2	116 ± 4.81	23.0 ± 3.08	13.8 ± 3.30
L. plantarum (B2994)	-	15.58 ± 0.74(++)	4.16 ± 0.04 ***	3.83 ± 0.01	133 ± 6.90	104 ± 8.77	29.6 ± 0.82	4.2 ± 2.76
L. casei (B2995)	-	12.18 ± 0.52(++)	8.36 ± 0.08 **	4.13 ± 0.08	96.5 ± 0.86	106 ± 3.33	21.5 ± 0.97	3.7 ± 2.17
L. plantarum (B2996)	-	16.05 ± 0.91(+++)	4.52 ± 0.58 ***	3.79 ± 0.04	128 ± 7.28	101 ± 3.35	24.7 ± 1.84	3.5 ± 0.11
L. buchneri (B2997)	-	5.11 ± 1.05(+)	11.74 ± 0.09 ^{NS}	5.13 ± 0.13	55.7 ± 5.63	116 ± 4.94	23.3 ± 4.08	9.5 ± 1.90
L. rhamnosus (B2998)	-	14.06 ± 1.06(++)	5.99 ± 0.39 ***	3.80 ± 0.01	144 ± 0.78	110 ± 6.52	24.3 ± 1.89	1.8 ± 0.56
L. bulgaricus (B2999)	+	8.64 ± 1.29(+)	11.84 ± 0.24 ^{NS}	4.82 ± 0.25	47.8 ± 1.58	120 ± 4.79	23.6 ± 2.59	9.7 ± 1.01
MRS (pH 5.8)[†]	-	-	11.44 ± 0.07	5.82 ± 0.17	0.52 ± 0.08	110 ± 0.42	22.2 ± 1.19	11.5 ± 0.14

[a] (-) negative, (+) positive; [b] (+++) inhibition zone >16 mm, (++) inhibition zone between 16-11 mm, (+) inhibition zone < 10mm; [c] * P<0.05, ** P<0.01, *** P<0.001, ^{NS} not significant; [d] Cell free supernatant; [†] control.

3.2.4 Selection and evaluation of probiotic and prebiotic candidates for synbiotic combination

3.2.4.1 Growth of *S.* Typhimurium in the presence of various prebiotics

The growth of STm SL1344nal^r in minimal medium (MM) supplemented with 1% of various prebiotics (LAC, FOS and GOS) was evaluated as described previously in section 2.1.9. The growth of STm SL1344nal^r with prebiotics as compared with that in MM supplemented with glucose showed that STm SL1344nal^r utilised the GOS mixture and FOS but not LAC (Figure 3.2). Area under the curve values for each substrate were 5.92 ± 0.082 (GOS), 2.46 ± 0.019 (FOS) and 0.33 ± 0.039 (LAC) and growth response compared against the glucose control was 254% (GOS), 60% (FOS) and 0% (LAC). The final OD readings at 24 hours were significantly lower (P<0.001) for LAC (OD 0.01 ± 0.001) and significantly higher (P<0.001) for GOS's (0.43 ± 0.005) compared to the glucose control (OD 0.21 ± 0.001), which was similar to the OD obtained with FOS (OD 0.20 ± 0.002).

A question arose regarding the response of STm SL1344nal^r to LAC in minimal medium. Was this 'no-growth' an inability to grow because the carbon and energy was not bioavailable due to the lack of the appropriate enzymic machinery to catabolise LAC, or, alternatively, was LAC inhibitory?

To test this, the experiments were repeated in a complex medium using standard lysogeny broth (LB) (Bertani, 2004) without glucose (LB-G) as the basal medium. The growth studies showed that STm SL1344nal^r grew in the presence of all prebiotics. Interestingly the AUC of 8.77 ± 0.059 obtained with LAC was the poorest in this test compared with the LB control with AUC of 9.71 ± 0.078 (Figure 3.3). The relative growth in LB medium containing test prebiotics was weakest for LAC at 90%, whereas utilisation of FOS and GOS resulted in growth response of 105% and 143%, respectively. The final OD readings at 24 hours were significantly lower (P<0.001) for LAC (OD 0.53 ± 0.002) and significantly higher (P<0.001) for GOS (0.86 ± 0.010) compared to the LB control containing glucose (OD 0.65 ± 0.002). There was no difference observed for the medium containing FOS (OD 0.66 ± 0.007). GOS used here significantly stimulated the growth of STm SL1344nal^r.

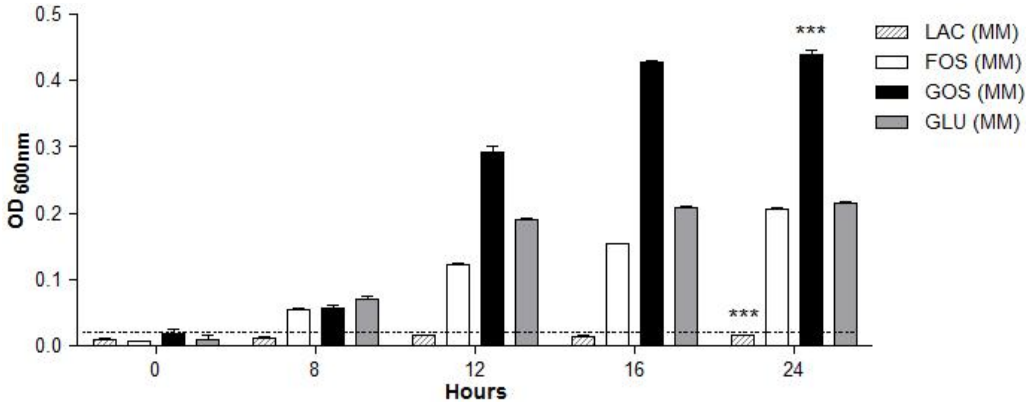


Figure 3.2 Growth of STm SL1344nal^r in minimal medium (MM) in the presence of 1% (w/v) LAC (hatched bar), FOS (white bar) and GOS (black bar). MM (grey bar) containing 1% (w/v) glucose was included as a control. Dotted line indicates *S.* Typhimurium SL1344nal^r growth in the presence of LAC. Cultures were incubated at 37°C for 24 hours and the OD was measured at 600nm (OD_{600nm}). The assay was performed in triplicate on three separate occasions and the SEM is shown. The OD values significantly different from the control at the final reading point are indicated by *** P<0.001.

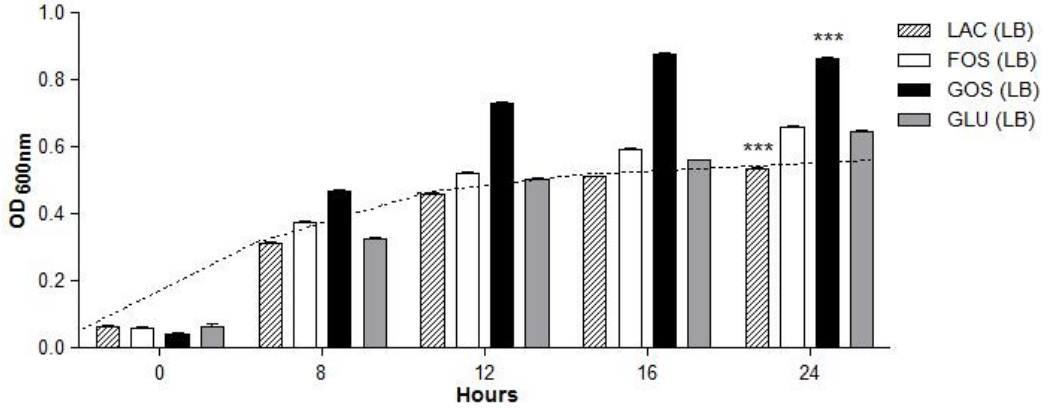


Figure 3.3 Growth of STm SL1344nal^r in LB-G the presence of 1% (w/v) LAC (hatched bar), FOS (white bar) and GOS (black bar). LB medium containing 1% (w/v) glucose (grey bar) was included as a control. Dotted line indicates *S.* Typhimurium SL1344nal^r growth in the presence of LAC. Cultures were incubated at 37°C for 24 hours and the OD was measured at 600nm (OD_{600nm}). The assay was performed in triplicate on three separate occasions and the SEM is shown. The OD values significantly different from the control at the final reading point are indicated by *** P<0.001.

3.2.4.2 Growth of lactobacilli in the presence of various prebiotics

Three out of sixteen *Lactobacillus* isolates (*L. plantarum* B2028, *L. plantarum* B2989 and *L. acidophilus* B2990) with the significant ($P<0.001$) antimicrobial activity against STm SL1344nal^r were assessed for their growth with prebiotics LAC, FOS and GOS (section 2.1.9). The growth as measured by AUC of each *L. plantarum* B2028, B2989, *L. acidophilus* B2990 on each prebiotic was calculated to its growth response on glucose which was set as 100%. The calculations were performed according to (Kneifel et al., 2000) and as detailed in section 2.1.9.

Using glucose as the growth standard (i.e./ 100%) the three lactobacilli showed similar growth on that LAC and GOS of 60%-80% and approximately 20% for FOS. The specific growth rates for all isolates were calculated as previously described (Saminathan *et al*., 2011) and demonstrated very similar results with no significant differences between strains on glucose and LAC (Table 3.3). Growth on FOS was relatively poor, but demonstrated higher growth rate for *L. plantarum* B2989 in comparison to *L. plantarum* B2028 and *L. acidophilus* B2990 ($P<0.05$; $P<0.001$, respectively). *L. acidophilus* B2990 demonstrated higher growth rates on GOS compared to *L. plantarum* B2028 and B2989 ($P<0.01$; $P<0.001$ respectively). For all three lactobacilli, the growth rate on LAC was slower in comparison to glucose (*L. plantarum* B2028 and B2989 $P<0.01$, *L. acidophilus* B2990 $P<0.05$).

The final OD_{600} values on this prebiotic for B2028, B2989 and B2990 were 1.03, 1.02, 1.13 respectively, showing that *L. acidophilus* reached a higher cell density at the final stage in comparison to both *L. plantarum* strains ($P<0.05$). *L. acidophilus* B2990 growth pattern was however characterised by longer lag phase comparing to *L. plantarum* strains. FOS was utilised poorly by all three lactobacilli with the final OD_{600} value following 24 hour incubation not extending above 0.20 and low growth rate values compared to glucose ($P<0.001$). The growth rate on GOS in contrast to glucose was significantly slower for *L. plantarum* B2028 and B2989 strains ($P<0.001$) and comparable for *L. acidophilus* B2990. The OD_{600} value reached following 24 hour incubation for those isolates were 0.84, 0.82 and 1.01 respectively.

Table 3.3 Growth parameters of *L. plantarum* B2028 and B2989, *L. acidophilus* B2990 in the presence of various prebiotics. Cultures were incubated at 37°C for 24 hours and the OD was measured at 600nm (OD_{600nm}). The assay was performed in triplicate on three separate occasions and the SEM is shown.

Substrate[a]	**Mean AUC**	**GR (%)**[b]	**GR (μ)**[c]	**OD_{600nm} at 24 h**
***L. plantarum* B2028**				
GLU[†]	14.75 ± 0.28	-	0.144	1.17 ± 0.04
LAC	11.29 ± 0.23	76.54	0.126	1.03 ± 0.01
FOS	3.17 ± 0.16	21.44	0.041	0.17 ± 0.01
GOS	11.07 ± 0.45	74.98	0.100	0.84 ± 0.02
***L. plantarum* B2989**				
GLU[†]	15.05 ± 0.03	-	0.143	1.19 ± 0.03
LAC	10.96 ± 0.24	72.93	0.119	1.02 ± 0.01
FOS	3.61 ± 0.08	18.76	0.051	0.20 ± 0.01
GOS	10.77 ± 0.19	71.46	0.095	0.82 ± 0.04
***L. acidophilus* B2990**				
GLU[†]	13.21 ± 0.32	-	0.146	1.11 ± 0.01
LAC	8.70 ± 0.38	67.09	0.132	1.13 ± 0.02
FOS	2.48 ± 0.21	18.76	0.032	0.14 ± 0.02
GOS	11.52 ± 0.17	87.30	0.153	1.01 ± 0.02

[a] GLU, LAC, FOS and GOS added as 1% (w/v) into basal modified MRS.

[b] Lactobacilli growth response (%) calculated as growth measured by AUC on each prebiotic to its growth on glucose (Kneifel *et al*., 2000).

[c] Specific growth rates on each prebiotic were calculated as previously proposed (Saminathan *et al*., 2011).

[†]Positive control set as 100%.

3.2.5 The effect of inclusion of the candidate prebiotics and *L. plantarum* B2028 in a porcine batch culture system upon bacterial numbers and SCFA concentrations

The porcine batch culture model employed to evaluate the inclusion of the synbiotic combination on the porcine faecal microbiota, mainly lactobacilli and bifidobacteria and the short chain fatty acid (SCFA) concentrations was set up as described in section 2.1.14 and as previously established (Martin-Pelaez *et al*., 2008; Sarbini *et al*., 2011; Saulnier *et al*., 2008). For these studies two synbiotic combinations were selected and comprised *L. plantarum* B2028 with either LAC or FOS. All batch fermenters containing basal medium (section 2.1.14) were inoculated with 10% (v/v) prepared faecal slurry and a control vessel (CTR) was left without the inclusion of a pre or synbiotic.

The results demonstrated that the number of bacteria detected with the Bif164 fluorescent *in situ* hybridisation (FISH) probe were not affected by the fermentation of LAC or FOS with or without the addition of *L. plantarum* (P=0.842). Furthermore, no changes were observed in total numbers of bacteria present which were detected with DAPI stain with all treatments (P=0.744). However, significantly increased numbers of bacteria in comparison to control were detected with the Lab158 probe with the mean values calculated from all collected time points for LAC, FOS, Lp + LAC, Lp + FOS and CTR (8.18 ± 0.29 (P<0.05), 8.19 ± 0.18 (P<0.05), 8.52 ± 0.17 (P<0.01), 8.05 ± 0.19 7.25 ± 0.06 log10 cells/ml respectively). A significant increase after 5, 10 and 24 hours compared with 0 hour was observed for all treatment groups with exception for Lp + FOS at 5 hours (Figure 3.4). In fermentation with CTR the number of bacteria remained similar after 5 hours and had decreased significantly at 24 hours. Taking into account different prebiotics and synbiotics at the specific time points, significantly higher numbers of bacteria were detected with the Lab158 probe was observed for LAC with regards to FOS at 10 hours of fermentation (P<0.01). Inclusion of FOS resulted in higher numbers of bacteria detected with the Lab158 probe than inclusion of synbiotic containing FOS at 10 hours. Moreover, at 5 hours, LAC and Lp + LAC showed higher count of *Lactobacillus-Enterococcus* in comparison to synbiotic (Lp + FOS) (P<0.001). For Lp + LAC significantly higher counts were also observed when compared to FOS and Lp + FOS (P<0.001) at 10 and to Lp + FOS at 24 hours (P<0.01).

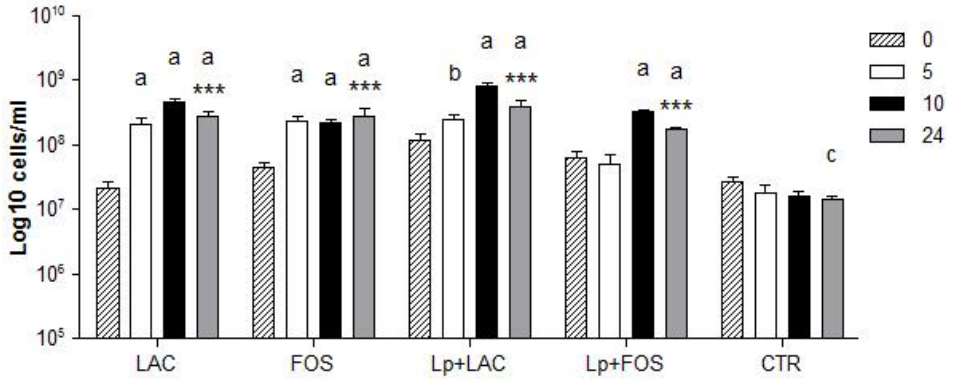


Figure 3.4 Bacterial numbers (Log10 cells/ml) enumerated using the Lab158 FISH probe over 24 hours incubation period within porcine batch culture system inoculated with LAC, FOS, *L. plantarum* B2028 + LAC (Lp + LAC), *L. plantarum* B2028 + FOS (Lp + FOS) and CTR. CTR was included inoculated only with 10% (v/v) faecal slurry. Bacterial numbers are showed at specific time points: 0 hour (hatched bar), 5 hours (white bar), 10 hours (black bar) and 24 hours (grey bar). The assay was performed in duplicate on two separate occasions and the SEM is shown. Values significantly different from the CTR at the 24 hours are indicated by *** $P<0.001$; values significantly different from the 0 hour at 24 hours are indicated by $^{a}P<0.001$, $^{b}P<0.05$ and $^{c}P<0.05$.

With the increased number of bacteria detected with the Lab158 probe, indicating increased numbers of lactic acid bacteria, it might be anticipated that there would be a concomitant increase in the production of their end product metabolites, and specifically lactic acid. To test this, samples were taken from each batch culture and subjected to HPLC analysis. Results showed that a significant and rapid increase in the lactic acid concentrations was observed between 0 and 5 hours and this was associated with the fermentation of FOS and inclusion of Lp + FOS ($P<0.01$; $P<0.05$, respectively). In comparison to CTR, concentrations of lactate for FOS and Lp + FOS also differed significantly at the 5 hours time point ($P<0.05$; $P<0.01$, respectively). Further incubation resulted in the decrease in lactic acid concentrations by 24 hours to a values not exceeding 1.5 mM for all conditions and showing no significant difference to CTR. The concentrations (mM) of lactic acid during the 24 hours incubation time with prebiotics and synbiotics are shown in Figure 3.5.

Inclusion of both prebiotics and synbiotics resulted in the gradual and significant increase in SCFA (sum of acetic, propionic and butyric acids) concentrations from 0 to

24 hours time point. Significant increase in SCFA was observed at 10 hours in comparison to CTR for FOS and Lp + FOS (P<0.01; P<0.001, respectively). At 24 hours the increase in SCFA concentrations in comparison to CTR was shown for all conditions (P<0.001) (Figure 3.6). Interestingly, the SCFA concentrations for Lp + FOS were significantly higher at 10 and 24 hours in comparison to Lp + LAC (P<0.05).

At time point 0 hour the two main detectable end product metabolites in the porcine batch culture system were lactic and acetic acids and those were found at concentrations below 2 mM for all the conditions. Following 5 hours incubation a gradual but slow increase in acetic acid concentration was observed for all conditions. A pronounced rise in lactate concentrations was observed, mainly for FOS and Lp + FOS, which were significantly higher in comparison to acetate (P<0.05; P<0.01 respectively). Further, a significant increase in acetate concentrations for Lp + FOS at 10 hours (P<0.05) was observed in comparison to CTR. Interestingly, the increase of acetate was correlated with decreases in lactate concentrations (P<0.01). Following the 24 hours incubation significant increase in SCFA was visible for all conditions (Figure 3.7).

For all conditions except Lp + LAC, acetate concentrations were significantly higher in comparison to CTR (P<0.001) after 24 hours. At the same time point propionate concentrations were significantly higher for all conditions in comparison to CTR (P<0.001; P<0.05 for Lp + LAC). Similarly for butyrate, higher concentrations were detected in comparison to CTR. After 24 hours fermentation, the acetic acid concentrations were significantly higher versus those of propionate for LAC and FOS (P<0.05) and significantly higher to butyrate for all conditions except the Lp + LAC synbiotic. Interestingly for LAC the concentrations of butyrate after 24 hours were significantly higher when compared with FOS or Lp + FOS (P<0.05; P<0.01). Moreover, the butyrate concentrations generated in Lp + LAC condition were significantly greater to those in Lp + FOS (P<0.05).

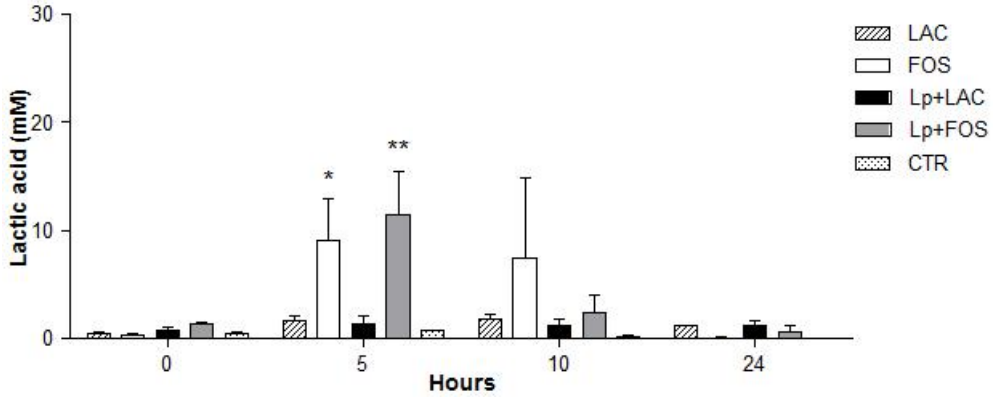


Figure 3.5 Concentrations (mM) of lactic acid at 0, 5, 10 and 24 hours of fermentation period, in a porcine batch culture system inoculated with LAC (hatched bar), FOS (white bar), *L. plantarum* B2028 + LAC (grey bar), *L. plantarum* B2028 + FOS (black bar). CTR (dotted bar) was inoculated only with 10% (v/v) faecal slurry. Prebiotics were included at 1% (w/v). The assay was performed in duplicate on two separate occasions and the SEM is shown. Values significantly different from CTR at the specific time point are indicated by * $P<0.05$ and ** $P<0.01$.

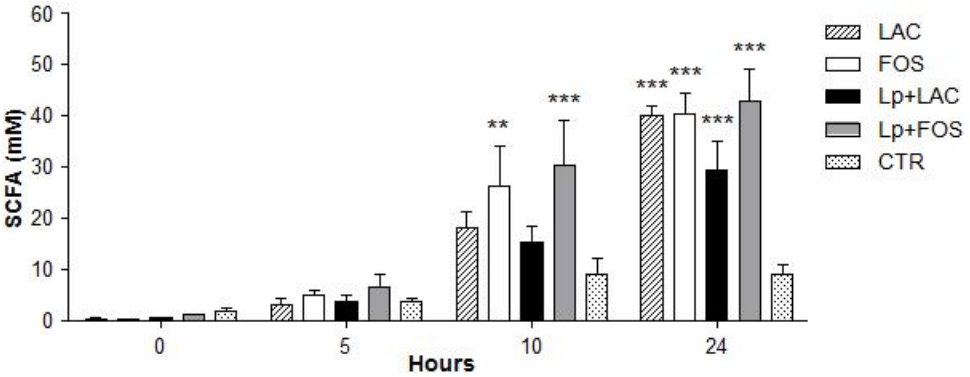


Figure 3.6 Concentrations (mM) of total SCFA (sum of acetic, propionic and butyric acid) at 0, 5, 10 and 24 hours of fermentation period, in a porcine batch culture system inoculated with LAC (hatched bar), FOS (white bar), *L. plantarum* B2028 + LAC (grey bar), *L. plantarum* B2028 + FOS (black bar). CTR vessel (dotted bar) was inoculated only with 10% (v/v) faecal slurry. Prebiotics were included at 1% (w/v). The assay was performed in duplicate on two separate occasions and the SEM is shown. Values significantly different from CTR at the specific time point are indicated by ** $P<0.01$ and *** $P<0.001$.

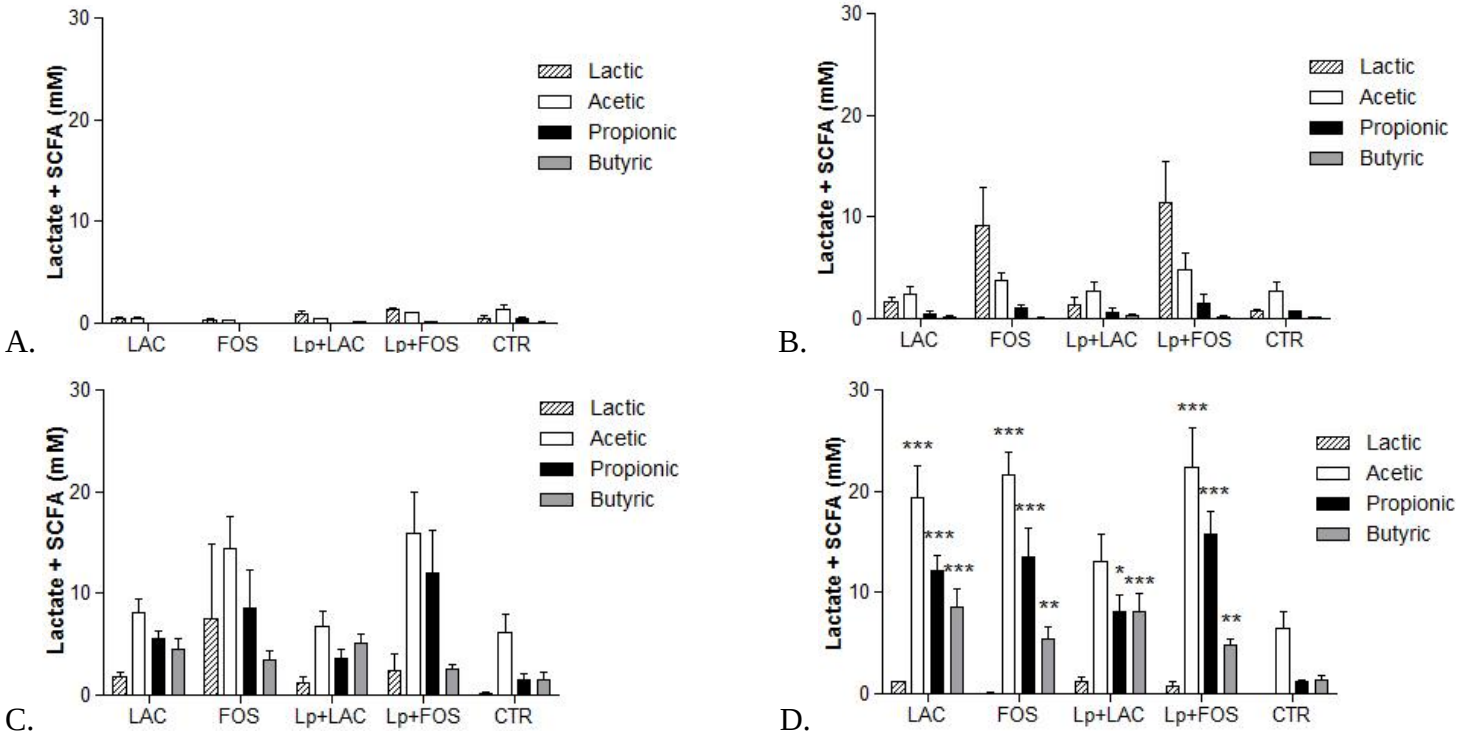


Figure 3.7 Concentrations (mM) of lactic (hatched bar) acetic (white bar), propionic (black bar) and butyric acid (grey bar) at times 0 hour (A), 5 hours (B), 10 hours (C) and 24 hours (D) hours in porcine batch cultures inoculated with prebiotics (LAC, FOS) and synbiotic combinations (*L. plantarum* B2028 + LAC, *L. plantarum* B2028 + FOS). CTR vessel was inoculated only with 10% (v/v) faecal slurry. Prebiotics were included at 1% (w/v). The assay was performed in duplicate on two separate occasions and the SEM is shown. Values significantly different from the CTR at 24 hours are indicated by * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

3.3 Discussion

Lactobacilli have been widely used as a feed supplements due to their health-promoting benefits both in human and animals. Therefore, the aim of this study was to assess combinations of probiotic and prebiotic, namely synbiotic combinations, for their potential to suppress the growth of a test pathogen, STm SL1344nal^{r}, in a surrogate pig intestinal model. However, to undertake *in vitro* studies to select the components of the synbiotic, a panel of representative pig *S.* Typhimurium isolates and other experimentally well defined experimental strains were to be used. Firstly, it was essential to confirm the identity of the bacterial isolates to be used in these studies. *Salmonella* isolates were obtained from the AHVLA national veterinary *Salmonella* reference library. The isolates were reconfirmed by Gram stain and biochemical tests to be *Salmonella* spp. and as serovar Typhimurium by standard laboratory slide agglutination test. From the panel of *Salmonella* strains, a nalidixic acid resistant derivative of *S.* Typhimurium SL1344 was used throughout this study to allow selective recovery for bacterial enumeration from complex experimental environments during *in vitro* and *in vivo* studies.

A panel of lactic acid bacteria isolates was available from the AHVLA culture collection and each came with a presumptive identification that was to be confirmed. The lactobacilli are notoriously difficult to speciate and this is why a battery of tests was used to give definitive descriptions of the isolates. All isolates were identified initially as lactobacilli based on their morphology, staining characteristics and catalase and oxidase reactions being coccal/rods, Gram+ve microorganisms. The identification of lactic acid bacteria has been based traditionally on their carbohydrate fermentation pattern. Using the API system to assess this, only moderate correlation of between 47% to 99.9% with presumptive identification was achieved, but in some instances there was no apparent correlation. This system failed to differentiate between closely related species (*L. casei* as *L. paracasei*). Other authors (Kwon *et al*., 2004; Yin and Zheng, 2005) have shown similar discrepancies when using carbohydrate fermentation profiles for identification purposes. For many lactic acid bacteria, mass spectral identification has proven to be a useful additional tool for a rapid identification at the species level (Angelakis *et al*., 2011; Tanigawa *et al*., 2010; Teramoto *et al*., 2007). However, correct identification of closely related species often fails. For example in this study, *L. casei* was identified as *L. paracasei*, one of the *L. rhamnosus* as *L.*

casei/*L. zeae* and *L. acidophilus* as *L. ultunensis*/*L. helveticus*. Similar discrepancies in speciation of closely related bacteria have been previously reported (Angelakis *et al*., 2011), when MALDI-TOF MS was used for the identification of lactobacilli from probiotic food and yoghurts. It has been suggested that both phenotypic and molecular characterisation should be employed to allow accurate identification of lactobacilli, particularly as discussed in closely related species (Holzapfel *et al*., 2001; Klein *et al*., 1998). Therefore, to assure a reliable identification both multiplex PCR and sequencing were used in this study. With the use of multiplex PCR (Kwon *et al*., 2004) it was possible to accurately identify six probiotic species, as it was designed for. The analysis of the 16S rRNA gene sequences showed match similarity 97-100% with the GenBank sequences, which together with other speciation methods used allowed confirmation of identity of each tested isolate at the species level. In conclusion, it was evident that no one method was universally capable of yielding accurate speciation. Thus, a combination of results was used. Where the majority of methods corroborated the original source description, this was taken as the species designation. Based on this approach, it was clear that using this battery of tests was essential for the identity of each isolate to be confirmed.

A number of studies have reported the antagonistic properties of lactic acid bacteria against various gastrointestinal pathogens (Collins *et al*., 2009). Some aspects of *Lactobacillus* spp. anti-pathogen activity are associated with the production of lactic acid, bacteriocin-like molecules, hydrogen peroxide and other unknown molecules (Servin, 2004). In order to select appropriate candidates for future research, the panel of lactobacilli strains was examined for their anti-*Salmonella* activity. Results generated from conditioned medium experiments in which the growth medium for STm SL1344 nal^r was supplemented with un-buffered CFS showed that *L. plantarum* B2028, *L. plantarum* B2989 and *L. acidophilus* B2990 exhibited the strongest inhibitory effect ($P<0.001$). There was a clear correlation between the extent of inhibition of the growth of STm SL1344nal^r on the one hand and the concentration of the lactic acid present and the pH of the cell free supernatant on the other. The production of lactic acid varied between species but also in between strains, which has been reported previously (Koll *et al*., 2008). All *L. plantarum*, *L. rhamnosus* and *L. casei* produced high concentrations of lactic acid, whereas only one of two *L. acidophilus* spp. showed strong antimicrobial activity and high lactic acid concentrations. Another mechanism by which lactic acid bacteria confer their

antimicrobial activity is the production of hydrogen peroxide and its inhibitory effect on *S*. Typhimurium has been demonstrated previously (Pridmore *et al*., 2008; Watson and Schubert, 1969; Yap and Gilliland, 2000). In this study the ability of lactobacilli to produce hydrogen peroxide was also examined and only *L. bulgaricus* B2991, *L. acidophilus* B2090, *L. acidophilus* B2993 and *L. delbruckei* B2999 were able to do so.

The construction of the appropriate synbiotic combination is an important and complex process which requires the selection of the prebiotic that would benefit the probiotic (Saarela *et al*., 2000). One of the key characteristics of an effective prebiotic is it's fermentability by the microflora colonising the gastrointestinal system (GIT) and selectivity towards the growth/activity of beneficial/probiotic bacteria present in the GIT (Kolida and Gibson, 2007). Therefore the subsequent studies showed to be an important part of the characterisation aiming to select the prebiotic which would selectively stimulate only bacteria of interest, namely potential probiotic bacteria, whilst not being metabolised and used for growth and respiration by STm SL1344nal^r. Of many prebiotics, lactulose (LAC), fructooligosacharides (FOS) and galalactooligosacharides (GOS) have been widely studied in human and pig nutrition in order to expand the knowledge of their beneficial properties (Collins and Gibson, 1999; Kaplan and Hutkins, 2003; Kolida *et al*., 2002; Laerke *et al*., 2000; Tzortzis *et al*., 2005). Studies conducted by Martin-Pelaez *et al*. (2008) indicated beneficial effects on the porcine microbiota following the administration of 1% of various prebiotics with LAC among them. Taking into consideration this previously published data on LAC as a prebiotic, in the present studies 1% (w/v) of each tested prebiotic was used. The results demonstrated that in pure cultures where competition for bioavailability in mono-culture with either STm SL1344 nal^r or the lactic acid bacteria there was no issue as STm SL1344 nal^r was unable to utilise lactulose as a sole carbon source. This is understandable since the *ebg* operon required for lactulose utilisation is absent in *Salmonella* (AbuOun *et al*., 2009). Unlike LAC, the prebiotics FOS and GOS both stimulated the growth of *Salmonella* although growth on FOS was less than GOS and in comparison to the glucose control. These data generated here indicate that both commercial GOS and FOS contain carbohydrates that are available to STm SL1344nal^r as metabolites even though it lacks the relevant enzymes to cleave the complex GOS and FOS oligosaccharides. Indeed, the work of Searle *et al*. (2010) eloquently demonstrated that GOS is comprised of various fractions of increasing complexity with the least complex fraction containing monomeric galactose that is a

known substrate for catabolism by *Salmonella*. Given the aim of the synbiotic mixture is to prevent and even suppress the growth of *Salmonella*, it was deemed sensible to consider LAC as a potential prebiotic as this substance does not stimulate the growth of *Salmonella*. It would of course be interesting to evaluate the bioavailability of the minor fractions of GOS and FOS in the pig gut. It is likely that monomeric carbohydrates would be readily catabolised by many bacterial species and, due to the competition for this available substrate, may not stimulate *Salmonella* to grow in that environment. The growth of *Salmonella* in LB broth, a complex medium used commonly for the growth of Enterobacteriaceae, was affected by the various prebiotics but to the lesser extent than when grown in supplemented minimal media. In LB medium the main carbon source for *Salmonella* are recovered from oligopeptides and amino acids, hence the growth of the pathogen was not limited to the utilisation of prebiotic supplementation. Consequently, the LAC had no obvious impact on the growth of *Salmonella* in either LB or minimal medium; it certainly did not have an inhibitory effect on STm SL1344 nal^r.

As probiotics are often capable of both synthesising as well as catabolising complex chain carbohydrates, it might be anticipated that many of the panel of lactic acid bacteria used in this study would be able to utilise LAC, GOS and FOS as carbon and energy sources. In the tests described in this chapter, the lactobacilli utilised LAC, FOS and GOS showing the highest growth stimulation with LAC and GOS. The growth response of lactobacilli on LAC and GOS, calculated in comparison with the glucose as a control (100%), was in value of over 60%, whilst growth on FOS was not higher than 21.4%. The species and strain specific utilisation of FOS was previously reported (Kaplan and Hutkins, 2003). In our study the growth of all lactobacilli on FOS as determined by OD extinction value showed no values beyond OD 0.2. This is in agreement with previously cited studies and suggests that those isolates were not able to utilise FOS and the modest growth that was observed was most likely due to the presence of simple sugar traces in the commercial FOS used in this study. Moreover, the highest growth rates for *L. plantarum* isolates B2028 and B2989 were observed on LAC and for *L. acidophilus* B2990 on GOS (Table 3.3). In this study GOS was suitable for the growth of all lactobacilli tested. The efficacy of a prebiotic depends on its selective fermentation and growth stimulation of particular bacterial groups, mainly lactobacilli and bifidobacteria. Considering these data, it was postulated that LAC supports the growth of the three *Lactobacillus* isolates and is

likely to be a most suitable carbohydrate from those tested for synbiotic co-administration. While the growth of probiotic was well supported by both LAC and GOS, the later also supported the growth of STm SL1344nal^{r} and the latter was precluded from consideration for development as a component of this particulate synbiotic. The results of this study accord with previous finding of Martin-Pelaez *et al*. (2008) and therefore, what further justify the use of LAC in future studies. Moreover, as LAC was indeed fermented by all three probiotic candidates.

With regard to the probiotic for use with LAC in pig studies it seemed appropriate to select a porcine isolate *L. plantarum* B2028 for future research. The ability to ferment LAC and the porcine origin of this strain may imply its better survival in and colonisation of porcine gut. The studies by Collins *et al*. (2010) propose this to be the case and so a synbiotic comprising LAC and *L. plantarum* B2028 was selected for further investigation.

In addition to pure culture studies and to assess microbial changes and SCFA production relating to the synbiotic inclusion, a static porcine simulated gut fermentation system was used. Although more complex culture systems are available for studying bacterial population shifts (Macfarlane and Macfarlane, 2007), the static batch culture was chosen because it is economic, rapid, efficient and an ideal first screen model system. The system was inoculated with the prebiotic (LAC or FOS) on its own and the synbiotic combination with *L. plantarum* B2028. FOS was included in this study despite being poorly metabolized by *L. plantarum* B2028 in the pure culture because it could possibly be favored by other members of porcine faecal microflora considered beneficial for the host (*Bifidobacterium* spp.) as it has been previously reported (Bouhnik *et al*., 2004; Saulnier *et al*., 2008). Assessments were made in the concentration of SCFA produced by GC methods and in the number of generic LAB by FISH. Lactobacilli and bifidobacteria genera are part of the normal intestinal microflora and are commonly used as probiotics (Roberfroid, 2000). Enhancement of these beneficial bacteria and modulation of the microbiota population are postulated to not only have a positive effect on the host, but also the creation of an unfavorable niche for pathogens. Although as shown in this study FOS was not fermented specifically by the prospective probiotic strain in the pure cultures, it was included in the batch cultures as a positive control, because numerous *in vitro* studies have demonstrated enhancement of *Lactobacillus* and *Bifidobaterium* spp. by this prebiotic (Kaplan and Hutkins, 2003; McBain and Macfarlane, 1997; Wang and Gibson, 1993).

However, in contrast to these studies, we have not observed significant changes in the *Bifidobacterium* population with either of the experimental conditions and no differences were seen in total bacterial numbers detected with DAPI. Similarly, Mountzouris *et al*. (2006) did not seen beneficial effect on the bacterial populations of pig's large intestinal segments fed prebiotics, including FOS.

Porcine batch culture model contains a complex microbial environment into which the prebiotics and synbiotic pairs were introduced and in these studies an increase in *Lactobacillus-Enterococcus* numbers after 24 hours in comparison to 0 hour was observed for both prebiotics and synbiotics. Moreover, at 24 hours for all conditions the *Lactobacillus-Enterococcus* count was significantly higher compared to the untreated control. Interestingly, throughout the fermentation where LAC and *L. plantarum* + LAC were included, the numbers of bacteria detected with the Lab158 probe were significantly higher in comparison to FOS or its synbiotic pair. In fact, the synbiotic containing LAC was better able to stimulate the *Lactobacillus-Enterococcus* group across the entire incubation time. The mean counts calculated along the 24 hours, for *L. plantarum* + LAC were significantly higher to control ($P<0.001$), whereas for *L. plantarum* + FOS no difference in the mean values was observed. As previously demonstrated, FOS failed to stimulate *Lactobacillus-Enterococcus* bacteria but increased the count of *Salmonella* when included in the batch culture system (Martin-Pelaez *et al*., 2008). Significantly higher counts of bacteria detected with the Lab158 probe with LAC containing synbiotics suggesting that when FOS and *L. plantarum* was added, FOS (in agreement with the pure culture studies) was not utilised by our probiotic but by other members of porcine faecal inocula, whereas LAC was in addition selectively fermented by *L. plantarum*.

An increase in the numbers of lactic acid bacteria, following the administration of prebiotics has been shown to be directly correlated with increased concentrations of fermentation products, mainly acetate, propionate butyrate and lactate (Pan *et al*., 2009). In our study, significant increases in lactate production were detected after 5 hours of incubation and especially associated with the fermentations with FOS and *L. plantarum* + FOS ($P<0.01$; $P<0.05$, respectively). This was also linked with a significant increase in *Lactobacillus-Enterococcus* group for FOS treatment group only. Perhaps, as suggested by Sakata *et al*. (2003), indigestible oligosaccharides could to some extent be providing the energy for the bacterial ecosystem to increase SCFA production but may not promote the growth of specific probiotic species. Interestingly,

the same significant increase in *Lactobacillus-Enterococcus* with LAC and LAC containing synbiotic was not associated with an increase of lactic acid concentration at the same time point. In this simple assessment we have only evaluated counts of *Lactobacillus-Enterococcus*, *Bifidobacterium* and total bacteria. Nevertheless, it is known that in pure cultures lactic acid is the major metabolic product of not only lactobacilli, bifidobacteria and enterococci, but also streptococci, eubacteria, fusobacteria and clostridia (Duncan *et al*., 2004). Further assessment targeting other bacterial populations is warranted.

The concentrations of lactic acid decreased to values not above 1.5 mM for all conditions after 24 hours. In the gut, lactic acid often plays a role of an intermediate metabolite and it is utilised by other bacterial species such as *Selenomonas, Veillonella* and *Megasphaera* and converted to acetate, propionate and butyrate (Duncan *et al*., 2004; Hashizume *et al*., 2003; Ushida *et al*., 2002). In our study a gradual increase in SCFA concentrations was observed during the first 5 hours and further significant increase in SCFA concentrations for FOS and *L. plantarum* + FOS at 10 hours ($P<0.01$, $P<0.001$) was observed in comparison to control. This SCFA increase was correlated with lactate concentrations decrease ($P<0.01$). Fermentation of both prebiotics resulted in significant increase in SCFA concentrations at 24 hours in comparison to the un-supplemented control. Interestingly, fermentation of LAC resulted in concentrations of butyrate after 24 hours that were significantly higher when compared with FOS or FOS containing synbiotic ($P<0.05$; $P<0.01$). Moreover, the butyrate concentrations generated in *L. plantarum* + LAC were significantly greater to those in *L. plantarum* + FOS ($P<0.05$). It has been shown that various butyrate producing genera such as *Clostridium*, *Eubacterium, Fusobacterium* can either convert lactic acid or even degrade prebiotics to butyrate as an end product (Belenguer *et al*., 2006; Bourriaud *et al*., 2005; Macfarlane and Macfarlane, 2007). Furthermore, the important role of *Megasphaera elsdenii* in lactate conversion to butyrate in the pig and cow has been pointed out (Counotte and Prins, 1981; Counotte *et al*., 1981; Ushida *et al*., 2002). In the studies by Tsukahara *et al*. (2006) inclusion of lactate-utilising *Megasphera elsdenii* in combination with lactic acid bacteria significantly stimulated the production of butyrate in the pig cecal *in vitro* model. A weakness of the current study was the lack of probing for these other relevant species. However, it should be remembered that this model system was a static fermentation rather than continuous flow and, as might be anticipated, the observations of SCFA

profiles is dynamic reflecting the evolution of the existing population at the start of the experiments and not, therefore, a reflection of the quasi steady state in the *in vivo* model. The stimulation of different bacterial populations by the specific prebiotics and lactate metabolism may be a partial explanation as to why in the present studies we did not observe a significant increase in lactic acid production with the lactulose fermentation. As the system is a static fermentation chamber, it is probable that other organisms utilise the SCFA acid and convert it to alternative end products. It would have been useful to use other probes for other genera such as *Clostridium*, *Eubacterium*, *Fusobacterium* and *Megasphaera* to assess this hypothesis. Bergman (1990) has stated that the beneficial role of SCFA to the health of the host, is due to supporting epithelial cell growth, blood flow and secretory and absorptive function of the large intestine.

Furthermore, recent studies have shown that even low concentrations of butyric and propionic acid decreased virulence gene expression of *S.* Typhimurium and subsequently decreased intestinal colonisation of pigs (Boyen *et al*., 2008). Fermentation of LAC with or without *L. plantarum* enhanced butyrate concentrations and generated lower concentrations of acetic acid in comparison to other conditions, and the latter has been known to upregulate virulence gene expression of *Salmonella* (Van Immerseel *et al*., 2004). Observations in this study of enhanced *Lactobacillus-Enterococcus* spp. and concentrations of butyric acid upon LAC fermentation was in agreement with the previously referenced study (Martin-Pelaez *et al*., 2008). In the same study, fermentation of FOS by porcine faecal microbiota was correlated with higher *S.* Typhimurium counts. Thus results generated in this section suggested that *L. plantarum* B2028 and LAC might be a more ideal synbiotic for further investigation as an intervention for *S.* Typhimurium infection in pigs.

Chapter 4

Evaluation of *L. plantarum* B2028 resistance to environmental stresses and its safety assessment as a part of further *in vitro* screening for a prospective probiotic

4.1 Introduction

Currently, the concept of alternative treatment strategies to control gastrointestinal pathogens such as *S.* Typhimurium in livestock is a popular area of research. In order to replace antimicrobials, probiotics, prebiotics, CE cultures and organic acids are well accepted as alternatives (Callaway *et al*., 2008; Cho *et al*., 2011; Papatsiros *et al*., 2012). In pigs, probiotic bacteria have not only been used to control microbial balance in disease control, but also as growth-promoters, improving efficiency of digestion (Cho *et al*., 2011; Collins *et al*., 2009). It has been postulated that probiotics improve intestinal balance resulting in better animal health, better growth and feed utilisation (Alexopoulos *et al*., 2004; Suo *et al*., 2012). It was also demonstrated that administration of lactobacilli strains lead to facecal microbial composition changes in young piglets which could be beneficial for the animal (Pieper *et al*., 2009; Simpson *et al*., 2000). Yet, in practice confirmation and identification of the specific health benefits of probiotics is not an easy task and has been interfered by various factors (Klaenhammer and Kullen, 1999).

The mechanism by which probiotics exert their anti-pathogenic effect is multifactorial including for example production of organic acids, bacteriocins, hydrogen peroxide, via competition for nutrients (Coconnier *et al*., 1997; Collins *et al*., 2009; Corcionivoschi *et al*., 2010) but these are not yet fully identified. However, firstly, in order to colonise the GI tract and exert their functional properties, probiotics must survive passage through the gastric environment of the stomach, withstand the antibacterial properties of bile and preferentially adhere to intestinal epithelium

(Jankowska *et al*., 2008; Klaenhammer and Kullen, 1999; Pan *et al*., 2008) and selection of strains which display these traits is highlighted (Fuller, 1989).

As for any feed additive, probiotics are tightly regulated, and currently within the EU the approval and risk management of a probiotic product is the obligation of the European Commission (EC) and its constituent member states (von Wright, 2005). Subsequently, feed additives including probiotics must adhere to guidelines covered in Council Directive 87/153/EEC (Anadon *et al*., 2006). The requirements for a microbial feed additive cover its clear identification, characterisation to the species level, evidence of probiotic efficacy and tolerance by the animal. Furthermore, it must be safe for the operator and it must not pose a risk to the safety of the end-consumer (SCAN, 2001). Council Directive 87/153/EEC states that "viable micro-organisms used as an active agent(s) in fed additives should not add to the pool transferable antibiotic resistance genes already present in the gut" (EFSA, 2005; SCAN, 2001; von Wright, 2005). Thus in addition, it is mandatory to assess antibiotic sensitivities and deduce the genetic basis of any resistance prior to its clinical use. The possibility of the resistance genes to exchange is not the only concern which encourages evaluation of lactobacilli resistance profile, but also the need of antibiotic therapy when *Lactobacillus* associated infections occurs (Cannon *et al*., 2005; Salvana and Frank, 2006) although exceedingly rare.

The data presented in the previous chapter enabled the selection of a prospective synbiotic combination which is to be tested as a control strategy for *S*. Typhimurium colonisation in pigs. As briefly mentioned above, in order to be able to exert its beneficial effects, a successful potential probiotic strain is expected to have a numerous of desirable properties and many reports list the selection criteria that should be applied (Klaenhammer and Kullen, 1999; Saarela *et al*., 2000). It was demonstrated in the previous chapter that *L. plantarum* B2028 exerts strong antimicrobial effect against *S*. Typhimurium growth. Therefore, in an attempt to address other selection criteria before the animal study and to conform with the EU legislative framework, the tolerance of *L. plantarum* B2028 to acid and bile, its adherence to epithelial cells and the antimicrobial susceptibility is discussed in this chapter.

4.2 Results

4.2.1 Evaluation of *L. plantarum* B2028 ability to resist environmental stress

4.2.1.1 Acid tolerance

The acid tolerance assay was performed according to previously described method by Hyronimus *et al*. (2000) (section 2.1.10). While *L. plantarum* B2028 was grown in MRS medium, it was washed in and subsequently inoculated into pH adjusted phosphate buffered saline (PBS). Results showed that the viability of *L. plantarum* B2028 decreased most rapidly at pH 2.0 with 3.22 ± 0.24 $\log_{10}$ reduction in bacterial numbers after 3 hours and with no viable cells recovered after 6 hours incubation. Similar decrease in the number *L. plantarum* cells was observed at pH 2.5 with the significant loss of viability observed within first 3 hours of incubation (P<0.001) (Table 4.1). However, only 0.03 ± 0.01 $\log_{10}$ reduction in the number of viable bacteria was observed during 3 hours of incubation at pH 3.0. Further incubation at pH 3.0 resulted in a 0.51 ± 0 $\log_{10}$ reduction, that was significantly different (P<0.01). A decrease in the number of viable cells was also observed when *L. plantarum* was tested in the non-adjusted 0.1M PBS (pH 7.2) with 0.11 ± 0.02 and 1.05 ± 0.12 $\log_{10}$ reduction at 3 and 6 hours respectively.

Table 4.1 The effect of acidic conditions on *L. plantarum* B2028 survival. *L. plantarum* numbers (Log_{10} cfu/ml) determined after 3 and 6 hours incubation at 37°C in 0.1M PBS at various pH values. The assay was performed in triplicate on three separate occasions and the SEM is shown. Values significantly different from 0 hour are indicated by **P<0.01 and *** P<0.001.

Condition	***L. plantarum* B2028 ($\log_{10}$ cfu/ml)**			**Log_{10} reduction**	
	0 h	**3 h**	**6 h**	**3 h**	**6 h**
pH 2.0	6.07 ± 0.01	2.85 ± 0.22***	< 1	3.22 ± 0.24	< 6
pH 2.5	6.06 ± 0.02	3.28 ± 0.18***	< 1	2.78 ± 0.20	< 6
pH 3.0	6.10 ± 0.01	6.06 ± 0.01	5.58 ± 0.01**	0.03 ± 0.01	0.51 ± 0
pH 7.2	6.10 ± 0.01	5.99 ± 0.01	5.04 ± 0.10***	0.11 ± 0.02	1.05 ± 0.12

4.2.1.2 Tolerance to bile

The tolerance of *L. plantarum* B2028 to bile was tested according to the previously established method (Gilliland *et al*., 1984) and as previously described in section 2.1.11.

Following the principles of Chateau *et al*. (1994), *L. plantarum* B2028 was sensitive to 0.3%, 0.6% (w/v) Oxgall salts and 0.9% (v/v) native porcine bile, with the delay of growth (*d*) greater than 60 minutes compared with MRS control (*d* > 60 minutes). The lower concentrations of native porcine bile demonstrated weaker inhibitory effect towards *L. plantarum* B2028 growth. The *L. plantarum* B2028 was weakly tolerant to 0.6% porcine bile and resistant to 0.3% porcine bile (*d* = 45, *d* = 15 respectively) (Table 4.2).

Table 4.2 Tolerance to 0.3%, 0.6% (w/v) Oxgall bile salts and 0.3%, 0.6% and 0.9% (v/v) native porcine bile of *L. plantarum* B2028. Cultures were incubated at 37˚C for 24 hours and the absorbance was read at OD_{600nm}. The assay was performed in triplicate on three separate occasions and the SEM is shown.

Bile type and concentration	Time to reach an OD of 0.3 at 600nm (hrs)	Delay of growth[a] (min)	Tolerance[b]	24h OD
MRS[†]	11.30 ± 0.04	-	+	1.11 ± 0.03
0.3% Oxgall	12.45 ± 0.03	> 60	-	0.90 ± 0.01
0.6% Oxgall	17.15 ± 0.04	> 60	-	0.36 ± 0.06
0.3% Porcine bile	11.45 ± 0.06	15	+	1.11 ± 0.02
0.6% Porcine bile	12.15 ± 0.04	45	±	0.97 ± 0.01
0.9% Porcine bile	13.25 ± 0.04	> 60	-	0.74 ± 0.03

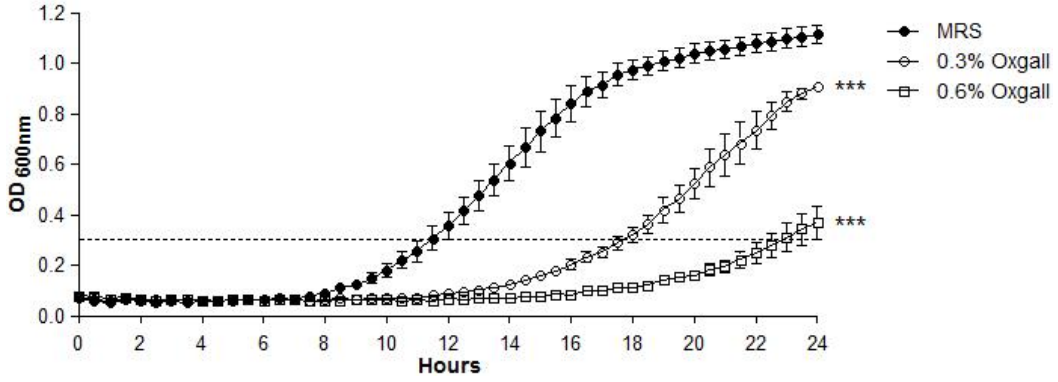
[a] Delay of growth (min) measured using optical density (OD_{600nm}) to increase by 0.3 units between MRS control and MRS containing bile.

[b] (+) resistant, (±) weakly resistant, (-) sensitive (Chateau *et al*., 1994).

[†] MRS control without bile.

The bactericidal effect of the Oxgall bile salts and porcine bile on the growth of *L. plantarum* B2028 was monitored for up to 24 hours. The growth of prospective probiotic showed to be significantly different with Oxgall or higher porcine bile concentrations when compared with MRS control ($P<0.001$) (Figure 4.1).

A.



B.

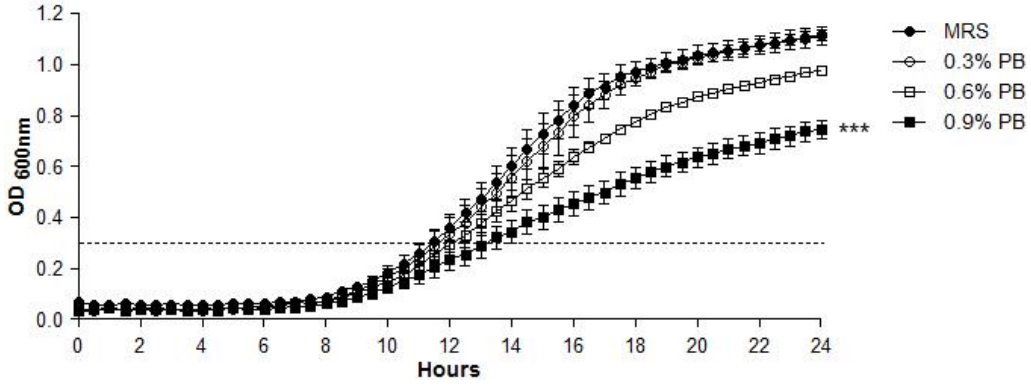


Figure 4.1 Growth of *L. plantarum* B2028 in MRS medium (closed circles) and MRS containing 0.3% (open triangle), 0.6% (open circle) and 0.9% (open squares) of bile (**A** – Oxgall bile salts (w/v), **B** – native porcine bile (PB) (v/v)). MRS without bile was included as a control. Cultures were incubated at 37°C for 24 hours and the absorbance was read at 600nm (OD_{600nm}). Dotted line indicates OD = 0.3. The assay was performed in triplicate on three separate occasions and the SEM is shown. Values significantly different from MRS control growth over 24 hours is indicated by *** $P<0.001$.

Screening for bile salt hydrolase (BSH) activity of *L. plantarum* B2028 was undertaken, however, these preliminary results were inconclusive and further investigation would be required.

4.2.2 Adherence of *L. plantarum* B2028 to porcine epithelial cell line

The ability to adhere to the host epithelial cells was suggested to be an important property of many probiotic strains (Morelli, 2000). The assay was performed as described previously in section 2.5.2 and the number of *L. plantarum* B2028 adhered cells to IPEC-J2 monolayers with or without lactulose was determined after one and two hour's incubation period. The presence of 1% (w/v) LAC in the growth medium or tissue culture medium had no effect on the adherence of *L. plantarum* B2028 to IPEC-J2 monolayers. As anticipated, the number of probiotic bacterial cells adhering increased with time and after 2 hours was significantly different as compared with 1 hour incubation ($P<0.001$) (Figure 4.2).

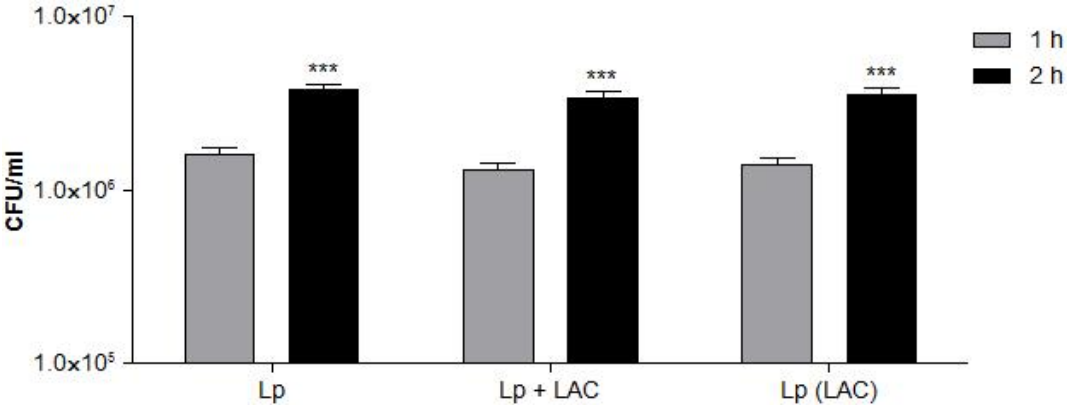


Figure 4.2 Adherence of *L. plantarum* B2028 to IPEC-J2 cells in the presence or absence of LAC, following 1 hour (closed, grey bar) and 2 hours incubation period (closed, black bar). The assay was performed in triplicate on three separate occasions and the SEM is shown. Values significantly different from the values of 1 hour incubation time point are indicated by *** $P<0.001$.

4.2.3 Evaluation of the candidate pre and probiotic cellular toxicity

Giemsa staining was carried out as previously described in section 2.5.5 and slides were assessed for vacuolisation, nuclear condensation and cellular detachment using a light microscope and compared to uninfected cells. The results showed that *L. plantarum* B2028, *L. plantarum* B2028 and LAC induced minor vacuolization, also observed when CFS was included. However, for all conditions used no significant cellular pathology was recorded.

4.2.4 Further *L. plantarum* B2028 safety assessment - antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined as previously described in section 2.1.13. *L. plantarum* B2808 was tested for resistance to ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, streptomycin, tetracycline and vancomycin with the exception of quinupristin/dalfopristin. According to the FEEDAP Panel microbiological breakpoints (EFSA, 2008), *L. plantarum* B2028 was susceptible to ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, streptomycin and tetracycline. The MIC value for vancomycin for facultative heterofermentative lactobacilli was not specified. However, the breakpoint for homofermentative lactobacilli is 2ng/µl, demonstrating the resistance of *L. plantarum* B2808 to this antibiotic (MIC >256). Table 4.3 shows MIC breakpoints for *L. plantarum* B2808.

In addition to MIC determination, a previously developed Gram positive microarray (Perreten *et al*., 2005) was used as described in section 2.2.6. The control for the microarray consisted of biotin-labelled oligonucleotides which were positive. The microarray confirmed the presence of aminoglycoside resistance genes (*aac(6')-li*, *aac(6')-lm*, *ant(4')-la*, *aph(2")-lb*, *aph(3')-III*), streptothricin resistant gene (*sat4*), MLS_B resistance gene (*erm(B)*), tetracycline resistance genes (*tet(L)-1*, *tet(M)*, *tet(U)*) and vancomycin and teicoplanim resistance genes (*van(A)*, *van(Z)*) for *E. faecium* SF11770. Furthermore, the *E. faecium* was also positive for internal positive control. This preliminary result of the microarray for *L. plantarum* B2808 indicated an absence of resistance genes. However, for *L. plantarum* B2028 the array internal controls were negative which may indicate a failure to detect resistance genes. Future analysis using specific primers for PCR would be desirable to complete molecular screening for antimicrobial resistance in *L. plantarum* B2028.

Table 4.3 Minimum inhibitory concentration (MIC) results for *L. plantarum* B2028. MIC's were determined for all, except quinupristin/dalfopristin recommended antimicrobials (EFSA, 2008). The assay was performed in duplicate.

MIC breakpoints (ng/µl)				
	***L. plantarum* B2028**		***E. faecalis* ATCC 29212** [a]	
Antibiotic tested	**Actual**	**MIC breakpoint** [b]	**Actual**	**Expected**
Ampicillin	1	2	2	1
Chloramphenicol	8	8	8	4
Clindamycin	<0.06	1	16	8
Erythromycin	0.5	1	4	4
Gentamicin	1	16	16	8
Kanamycin	32	64	128	-
Streptomycin	16	n.r.	256	-
Tetracycline	16	32	16	16
Vancomycin	>256 [c]	n.r.	4	2

[a] MIC control

[b] Microbiological breakpoints defined by FEEDAP. Strains with higher that MICs breakpoint are considered resistant (EFSA, 2008).

[c] Intrinsic resistance to vancomycin reported as a general feature (Danielsen and Wind, 2003).

n.r.- not required for *L. plantarum* spp.

4.3 Discussion

The findings presented in the previous chapter aided the selection of the prospective synbiotic combination, which was ultimately subjected to further *in vitro* studies prior to its inclusion in an *in vivo* model. Nevertheless, the concern of 'suitability' of *L. plantarum* B2028 for animal model use and the ethical responsibility to assess risks prior to any animal studies, lead to the studies described in this chapter. Klaenhammer and Kullen (1999) functionally collated 'appropriate probiotics' into four general categories, (appropriateness, technological suitability, competitiveness, performance and functionality) listing the ability to resist the environmental stresses of gastric pH and bile and adherence within the GIT as a part of the competitiveness category. Efficacy of the probiotic will need to be validated ultimately within the *in vivo* model. However, the use of an array of *in vitro* tests is recommended, especially with regards to the safety of the probiotic.

Surviving passage through the stomach is important for the probiotic efficacy and resistance to low pH is considered as candidate desirable property. The viability of the isolate will depend on numerous factors such as present pH, the time of exposure (Bezkorovainy, 2001), the form and the strain type (Marteau *et al*., 1993). In general the pH of the porcine stomach is considered similar to that of humans with the pH ranging from as low as 1.15 to 4.0 (Hossain *et al*., 1990; Kass *et al*., 1980). However, the pH values of contents of the stomach are known to differ between anterior portion and the posterior portion (in the pig pH 4.3 and pH 2.2 respectively) (Kararli, 1995; Smith, 1965). Consideration needs also to be given to the matrix, such as food or protective formulation, in which the probiotic is presented to the gastric environment and the time of exposure to that environment. The transit time appears to be proportional to the size of the substance, but also the presence or absence of the food in the stomach (Hossain *et al*., 1990). It was demonstrated that gastric emptying in the pig consists of two phases, first a rapid phase occurring at the feeding stage and the second extended phase characterised by regular but slower emptying (Gregory *et al*., 1990). This process takes considerably less time in young pigs compared to adult (Kararli, 1995). Laboratory based screening showed the ability of *L. plantarum* B2028 to survive the 3 hours exposure to all tested pH ranges. However, with the lowest pH range (pH 2 and pH 2.5) over 2-log reduction in viable counts was observed. Further exposure, up to 6 hours to those pH values resulted in complete loss of viability.

Interestingly, *L. plantarum* B2028 was resistant to pH 3.0 and only after 6 hours of exposure 0.51-log reduction in viable counts was observed. Given the variable pH of the porcine stomach and relatively rapid gastric emptying rate (Gregory *et al*., 1990), it may be reasonably argued that a proportion of viable probiotic cells will reach the small intestine within the first 3 hours. Moreover, Corcoran *et al*. (2005) demonstrated that survival of *L. rhamnosus* GG in simulated gastric juice at pH 2.0 was improved in the presence of carbohydrates that could be metabolised by this strain. It might also be anticipated that administration of *L. plantarum* B2028 together with lactulose may enhance the probiotic candidate survival.

Prospective probiotics capable of surviving harsh acidic environment will further encounter the membrane damaging effect of bile during the passage through the small intestine (Bron *et al*., 2004). Bile consists of bile salts, cholesterol and phospholipids (Kararli, 1995). Cholic acid and chenodeoxycholic acid are the primary bile salts present in the human gut and a range of animal species (Coleman *et al*., 1979). The proportion of these primary bile acids in mammals and therefore toxicity depends on the species, as it is recognized that dihydroxy bile salt are more effective at membrane disruption than trihydroxy bile salts (Coleman *et al*., 1979; Vyvoda *et al*., 1977). Secondary bile acids like deoxycholic and hyodeoxycholic are by-products of former bile salts, produced by bacterial metabolism (Ridlon *et al*., 2006). In pigs, the primary bile acids are glycine and taurine conjugates of dihydroxy bile salts, with smaller quantities of trihydroxy bile salts (Coleman *et al*., 1979) and the most abundant secondary bile acid is hyodeoxycholic acid (Coleman *et al*., 1979; Haslewood, 1971). Considering the species specific composition of bile, in addition to the routinely used Oxgall bile salts, the tolerance of *L. plantarum* B2028 to fresh porcine bile collected from a gall bladder was evaluated here. This model was thought to be more representative of the porcine *in vivo* environment. According to the classification proposed by Chateau *et al*. (1994), *L. plantarum* B2028 would be considered sensitive to 0.3% bovine bile salts, but fully resistant to 0.3 - 0.6% of the porcine bile. Only 0.9% of the freshly collected porcine bile significantly reduced the growth of the isolate ($P<0.001$). As demonstrated, the resistance of *L. plantarum* B2028 towards Oxgall bile salts is low which could be due to differences in the bile composition. The resistance to (0.3-0.6%) porcine bile and the host origin indicates that *L. plantarum* may be able to resist the antimicrobial properties of bile. However, care should be taken with this argument as the concentration of bioavailable and bioactive bile acids

in Oxgall and the fresh porcine sample may not be the same.

In the intestine conjugated bile acids can be hydrolyzed by a range of bacteria present in the gut and among the various genera *Lactobacillus* is also found to express conjugated bile salt hydrolases (BSHs). These enzymes have been speculated to decrease the harmful effect of bile salts and, therefore, may be important for colonisation (Moser and Savage, 2001). Attempts were made to screen for BSH activity (data not presented) expressed by *L. plantarum* B2028 but the results were inconclusive. In hindsight, screening for the presence of the bile salt hydrolase *(bsh)* gene may have been an appropriate first step before undertaking detailed biochemical analyses to establish the phenotype. In conclusion, the survival studies presented here demonstrated the potential of *L. plantarum* B2028 to resist the stresses it might encounter in the upper alimentary tract of pigs and, therefore, this organism is likely to reach the colon assuming a sufficient inoculum is delivered orally to the pig. Only *in vivo* feeding trials will test this hypothesis.

The adherence of *Lactobacillus* isolates has been thought to enhance the likelihood of intestinal colonisation (Adlerberth *et al*., 1996; Klaenhammer and Kullen, 1999; Morelli, 2000). Moreover, the ability of probiotics to attach to intestinal cells is correlated with their capacity to interfere with the adherence of pathogens *in vitro* (Bezkorovainy, 2001). The results in this chapter demonstrated that *L. plantarum* B2028 was competent at adhering to porcine epithelial cells regardless of the presence or absence of LAC. It can be concluded that LAC did not influence the adherence of *L. plantarum* B2808 and the question remains whether LAC may inhibit the adherence of pathogens as shown for GOS by Searle *et al*. (2010). This issue will be tackled in the next chapter.

Much of the selection criteria for commercial probiotics are based on assessing safety. Probiotic organisms are generally defined as non pathogenic and beneficial to the host physiology (Ouwehand *et al*., 2002). However, in order to be used as a feed additive, probiotics must be classified as generally regarded as safe (GRAS) and therefore not cause any harmful effects on human or animal health (Tannock, 2003).

Firstly, we evaluated the cellular toxicity towards a porcine epithelial cell line of *L. plantarum* B2808, lactulose and CFS. Giemsa stain results showed the presence of only minor vacuolization following incubation with the bacteria, CFS and lactulose. Although severe and long term vacuolization can lead to the cell death, the process of vacuolization has been associated with the adaptive physiological response to a

stressor and it is thought to be fully reversible (Henics and Wheatley, 1999). The causes of vacuolization are multifactorial and it is difficult to give any clear reason why the minor vacuolization in the cell line was generated. Technically, monolayers by default are highly stressed cells and any further stress may induce the minor vacuolization that was observed. Nevertheless, healthy epithelial cells in their natural environment and with the presence of the mucus lining in the pig gut might suggest that our observations will not be translated *in vivo*.

Further stringent safety assessment is particularly important when taking into consideration the antibiotic resistance in these bacteria (Gueimonde and Salminen, 2006; Salminen *et al*., 1998). The extensive use of antimicrobials in both human and animal medicine resulted in the selection of antibiotic resistance (Davies, 1997; Mathur and Singh, 2005). Antibiotic resistance in bacteria can be either intrinsic or acquired. The intrinsic resistance is not generally horizontally transferable, therefore, creates less risk in non-pathogenic bacteria (Mathur and Singh, 2005). On the contrary, specifically acquired resistance (via plasmids, transposons or integrons carrying antibiotic resistance genes) is of significant interest as there are fewer obstacles for transfer between pathogenic, potentially pathogenic and commensal LAB what could lead to the exchange of the resistance genes (Teuber, 1999; Teuber *et al*., 1999). It is therefore obligatory that prior to clinical use probiotic strains need to be fully characterised in order to differentiate the antimicrobial resistant and sensitive strain. As pointed out previously, FEDDAP guidelines for determination of MIC's against a range of widely used antimicrobials are essential for the safety screening of probiotics. *L. plantarum* B2028 isolate was found to be susceptible to ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, streptomycin and tetracycline and resistant to vancomycin. Resistance to vancomycin has been seen in variety of lactobacilli, however the resistance pattern is not related to the type found in enterococci (Salminen *et al*., 1998; Tynkkynen *et al*., 1998). Elisha and Courvalin (1995) demonstrated that intrinsic resistance mediated by D-alanine: D-alanine ligase-related enzymes towards vancomycin in *Lactobacillus* spp. are not closely related to the Ddl-related enzymes, VanA and VanB associated with the acquired and therefore mobile resistance in enterococci. Moreover intrinsic resistance to vancomycin has been reported as a general feature for lactobacilli (Danielsen and Wind, 2003). In conclusion, the results obtained in this chapter would indicate that *L. plantarum* B2028 be considered susceptible to a range of commonly used antimicrobials. However, to

complete that screening the MIC for quinupristin/dalfopristin need to be carried out in future studies. The phenotypic data were in agreement with the molecular screening although some doubt must be cast over the array as the internal controls may have failed. In addition prior to commercial application of this strain screening for the presence of virulence factors should also be considered as an important aspect of safety evaluation.

Chapter 5

An *in vitro* evaluation of *L. plantarum* B2028 and lactulose antagonistic activity against *S*. Typhimurium

5.1 Introduction

Salmonella infections in animals, including pigs result in health, productivity and economic loses; in the EU anticipated cost associated with pork is around €90 million (Anonymous, 2010). Moreover, it has been recognised that pork and pork products are an important source of *Salmonella* infections in humans (Lo Fo Wong *et al*., 2002). Antimicrobial growth promoters have been used in animals since 1940's due to numerous health benefits including disease control (Page, 2006). However, increasing antibiotic resistance became recognised as a major problem that has implications for both human and animal health (McEwen and Fedorka-Cray, 2002; Threlfall, 2000) and this has led to their removal as feed additives in the EU in 2006 as regulated by the Regulation (EC) No 2160/2003 (Anonymous, 2003).

The concept of including prebiotics and probiotics in the animal diet is an area of current active research (Callaway *et al*., 2008) driven largely by the need to reduce dependency on the use of antimicrobials. Among various alternative control strategies applied in human and animals is the consumption of probiotics as this has been associated with health benefits in both (Fuller, 1989). The beneficial effects for the host are considered due to improved intestinal homeostasis and the inhibitory action of probiotics against pathogens that could be mediated via immunomodulation, enhancing epithelial barrier function, competition for the receptor sites on the host epithelium and competition for nutrients in the gut (Chichlowski *et al*., 2007; Cho *et al*., 2011; Makras *et al*., 2006; Oelschlaeger, 2010; Servin, 2004). Prebiotics, such as the complex oligosaccharides that are not metabolized by the host but are by bacteria such as the bifidobacteria, stimulate beneficial bacteria in the gut thereby enhancing probiotic

effects (Macfarlane and Macfarlane, 2007; Macfarlane *et al*., 2008; Tzortzis *et al*., 2005). Both probiotics and prebiotics and combinations, called synbiotics, have been advocated as potent intervention measures that reduce adhesion and invasion of pathogenic bacteria *in vitro* and *in vivo* (Coconnier *et al*., 1997; Hudault *et al*., 1997; Lin *et al*., 2008; Searle *et al*., 2009; Searle *et al*., 2010). Moreover, the production of short chain fatty acids (SCFA) such as lactate and acetate plus other antimicrobial substances by probiotic bacteria has been reported to be inhibitory to a wide range of pathogenic bacteria (Coconnier *et al*., 1997; De Keersmaecker *et al*., 2006; Makras *et al*., 2006; Vandenbergh, 1993). SCFA have not only been implicated to have an antagonistic effect towards *Salmonella* and other enteropathogens *in vitro* and *in vivo* (McHan and Shotts, 1993) but also play a role in colonic health specifically in colonocytes differentiation and proliferation, especially butyrate (Macfarlane *et al*., 2008).

One of the testable hypotheses of the work presented in this thesis is that a synbiotic combination containing *L. plantarum* B2028 and LAC would effectively reduce *S*. Typhimurium colonisation in the pig. Before aforementioned feeding control strategy is investigated using an animal model, adequate experimental models that adhere to the three R's principles need to be employed (Boyen *et al*., 2009; Russell and Burch, 1992). To do this, the interactions of STm SL1344 nal^r with the 'host' and in the presence of pre, pro and synbiotic, extensive *in vitro* porcine models have been utilised. To investigate the *in vitro* efficacy of *L. plantarum* B2028 and LAC against STm SL1344 nal^r the studies described in this chapter employed growth, viability, adhesion and invasion assays. In addition to porcine faecal batch culture studies described in Chapter 3, here the effect of *L. plantarum* B2028 and LAC inclusion into the system on STm SL1344 nal^r survival is described.

5.2 Results

5.2.1 Antimicrobial activity of *L. plantarum* B2028 cell-free supernatant against *S.* Typhimurium

5.2.1.1 Inhibition of *S.* Typhimurium growth, a role of low pH and lactic acid

The effect of cell-free supernatants from *L. plantarum* B2028 grown in MRS (CFS) and MRS containing LAC (MRSL) (CFSL) on the growth of STm SL1344nal^r was investigated using the conditioned medium assay described previously (section 2.1.8). The pH adjusted MRS and MRSL broth were included as pH controls, whereas MRS/MRSL lactic acid controls contained L- lactic acid (-LA) (Table 5.1 and Table 3.1 of Chapter 3). The growth of STm SL1344nal^r was measured at OD 600nm (Figure 5.1 and Figure 5.2 A&B) and the reduction of growth was determined by the area under the curve (AUC) over 24 hours (Table 5.2).

Inclusion of *L. plantarum* B2028 CFS and CFSL at pH 3.8 inhibited the growth of STm SL1344nal^r for approximately 10 hours, after which time growth occurred but at a reduced rate compared with LB-G, a significant growth reduction (P<0.001) (Figure 5.1). Furthermore, the growth of STm SL1344nal^r was also significantly reduced with MRS-LA/MRSL-LA (P<0.001) and MRS pH control given at pH 3.8 when compared with LB-G. Interestingly, it was also observed that STm SL1344nal^r growth in the presence of *L. plantarum* B2028 supernatants (CFS/CFSL) and lactic acid control media (MRS-LA/MRSL-LA) (pH 3.8) was significantly reduced compared with relevant MRS/MRSL pH controls (P<0.001).

When *L. plantarum* B2028 supernatants and control media were included at pH 4.5, the growth of STm SL1344nal^r was significantly reduced only in the presence of *L. plantarum* B2028 CFS, CFSL and one of the lactic acid controls (MRS-LA) when compared with the LB-G control (P<0.001, P<0.001, P<0.01 respectively). Moreover, lactic acid controls (MRS-LA/MRSL-LA) at pH 4.5 had no effect on the growth of STm SL1344nal^r when compared with respective pH controls (MRS/MRSL), while with *L. plantarum* B2028 supernatants (CFS/CFSL) the reduction of the growth of STm SL1344nal^r was still observed (P<0.01, P<0.001) (Figure 5.2 A&B).

At pH 7.2, none of the conditions tested influenced STm SL1344nal^r growth when compared to its growth in non-conditioned LB-G medium; in fact, modest increases in the growth of STm SL1344nal^r were observed with pH and lactic acid controls (P<

0.001). Very small, nevertheless significant reductions of growth of STm SL1344nal^r were observed with both *L. plantarum* B2028 supernatants (CFS/CFSL) and one of the lactic acid control media (MRSL-LA) (P<0.001, P<0.01 respectively).

Table 5.1 Concentrations (mM) of organic acids in the *L. plantarum* B2028 CFSL and MRSL broth. The assay was conducted in triplicate and the SEM is shown.

Condition	**pH**	**Lactic and SCFA concentrations (mM)**			
		Lactic acid	**Acetic acid**	**Butyric acid**	**Propionic acid**
MRSL[a]	5.81± 0.10	1.40 ± 0.29	88.0 ±4.13	10.9 ± 0.06	11.5± 1.48
CFSL[b]	3.89± 0.08	123± 9.25	80.4 ± 7.65	19.8 ± 2.82	6.5 ± 0.53

[a] MRS broth with 1% (w/v) lactulose.

[b] Cell free supernatant of *L. plantarum* B2028 when grown in MRSL broth.

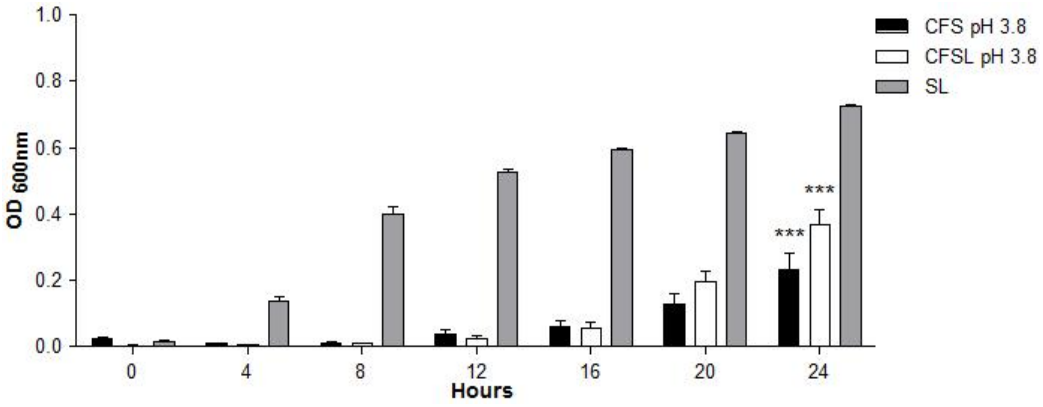
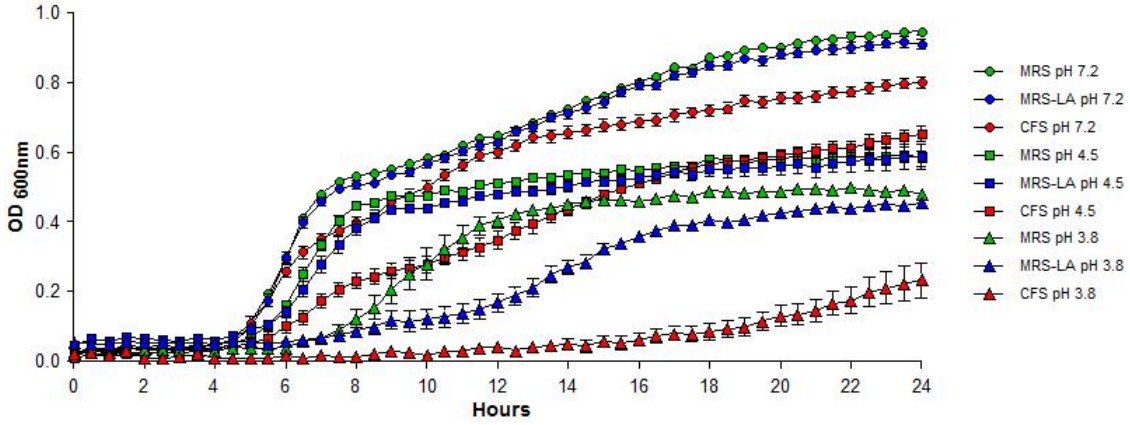


Figure 5.1 Growth of STm SL1344nal^r in LB-G broth (closed, grey bar) and with inclusion of 10% (v/v) *L. plantarum* B2028 CFS (closed, black bar) and CFSL (white bar). Cultures were incubated at 37°C for 24 hours and the OD was measured at 600nm (OD_{600nm}). The assay was performed in triplicate on three separate occasions and the SEM is shown. Values significantly different from the non-conditioned control (LB-G) at the final reading point are indicated by *** P<0.001.

A.



B.

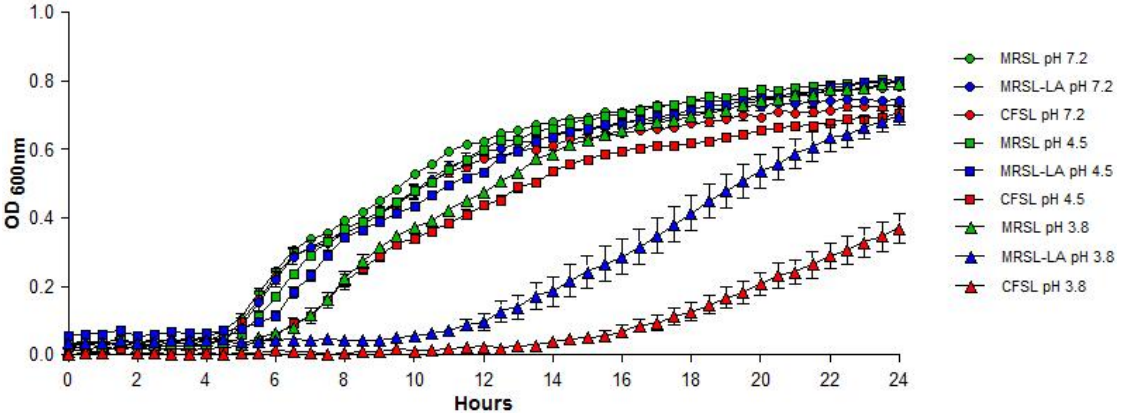


Figure 5.2 Growth of STm SL1344nal^{r} in LB-G broth supplemented with 10% (v/v) *L. plantarum* B2028 CFS (A) and CFSL (B) at pH 3.8 (red triangles), pH 4.5 (red squares) and pH 7.2 (red circles). Lactic acid controls (MRS-LA (A) and MRSL-LA (B)) adjusted to pH 3.8 (blue triangles), pH 4.5 (blue squares) and pH 7.2 (blue circles) and pH controls (MRS (A) and MRSL (B)) adjusted to pH 3.8 (green triangles), pH 4.5 (green squares) and pH 7.2 (green circles) were also included at 10% (v/v) dilution factor. OD at 600nm (OD_{600nm}) was measured as a growth reporter. The assay was performed in triplicate on three separate occasions and the SEM is shown.

Table 5.2 The growth of STm SL1344nal^r in the conditioned and pH controlled media.

Experimental condition	Mean AUC	Significance to LB-G control	Significance to pH control
S. Typhimurium SL1344 nal^r culture in LB-G (SL)‡	10.79 ± 0.16	-	-
SL + 10% (v/v) MRS[a] broth at pH 3.8 (MRS pH 3.8)[†]	7.09 ± 0.19	*P*< 0.001	-
SL + 10% (v/v) MRSL[b] broth at pH 3.8 (MRSL pH 3.8)[†]	10.11 ± 0.11	NS	-
SL + 10% (v/v) MRS broth containing lactate at pH 3.8 (MRS-LA pH 3.8)[††]	5.34 ± 0.26	*P*< 0.001	*P*< 0.001
SL + 10% (v/v) MRSL broth containing lactate at pH 3.8 (MRSL-LA pH 3.8)[††]	5.45 ± 0.62	*P*< 0.001	*P*< 0.001
SL + 10% (v/v) *L. plantarum* B2028 CFS[c] at pH 3.8 (CFS pH 3.8)	1.44 ± 0.31	*P*< 0.001	*P*< 0.001
SL + 10% (v/v) *L. plantarum* B2028 CFSL[d] at pH 3.8 (CFSL pH 3.8)	1.92 ± 0.23	*P*< 0.001	*P*< 0.001
SL + 10% (v/v) MRS broth at pH 4.5 (MRS pH 4.5)	9.59 ± 0.18	NS	-
SL + 10% (v/v) MRSL broth at pH 4.5 (MRSL pH 4.5)	11.55 ± 0.08	NS	-
SL + 10% (v/v) MRS broth containing lactate at pH 4.5 (MRS-LA pH 4.5)	9.20 ± 0.34	*P*< 0.01	NS
SL + 10% (v/v) MRSL broth containing lactate at pH 4.5 (MRSL-LA pH 4.5)	11.10 ± 0.17	NS	NS
SL + 10% (v/v) *L. plantarum* B2028 CFS at pH 4.5 (CFS pH 4.5)	8.11 ± 0.24	*P*< 0.001	*P*< 0.01
SL + 10% (v/v) *L. plantarum* B2028 CFSL at pH 4.5 (CFSL pH 4.5)	9.00 ± 0.17	*P*< 0.001	*P*< 0.001
SL + 10% (v/v) MRS broth at pH 7.2 (MRS pH 7.2)	13.70 ± 0.16	↑(*P*< 0.001)	-
SL + 10% (v/v) MRSL broth at pH 7.2 (MRSL pH 7.2)	11.87 ± 0.20	↑(*P*< 0.001)	-
SL + 10% (v/v) MRS broth containing lactate at pH 7.2 (MRS-LA pH 7.2)	13.26 ± 0.26	↑(*P*< 0.001)	NS
SL + 10% (v/v) MRSL broth containing lactate at pH 7.2 (MRSL-LA pH 7.2)	11.11 ± 0.12	↑(*P*< 0.001)	*P*< 0.01

Table 5.2 (cont'd).

Experimental condition	Mean AUC	Significance to LB-G control	Significance to pH control
SL + 10% (v/v) *L. plantarum* B2028 CFS at pH 7.2 (CFS pH 7.2)	11.65 ± 0.29	NS	$P < 0.001$
SL + 10% (v/v) *L. plantarum* B2028 CFSL at pH 7.2 (CFSL pH 7.2)	10.83 ± 0.08	NS	$P < 0.001$

[a] MRS broth containing 2% (w/v) glucose.

[b] MRS broth containing 1% (w/v) lactulose.

[c] *L. plantarum* B2028 cell free supernatant when grown in MRS broth.

[d] *L. plantarum* B2028 cell free supernatant when grown in MRSL broth.

† MRS /MRSL broth adjusted to relevant pH (3.8, 4.5, 7.2) used as pH control.

†† MRS /MRSL broth containing 145 mM and 123 mM L-lactic acid respectively and adjusted to relevant pH (3.8, 4.5, 7.2) used as lactic acid control.

‡ SL (*S.* Typhimurium SL1344 nal^r) grown in LB-G - non-conditioned medium control.

5.1.1.2 The effect of *L. plantarum* B2028 cell-free supernatant on viability and invasive phenotype of *S*. Typhimurium

Viability assays were performed following previously established procedures (Coconnier *et al*., 1997; Makras *et al*., 2006) with the slight modifications as described in section 2.5.3. No significant reduction of STm SL1344nal^r viability was observed after 1 hour of incubation with 10 % (v/v) MRS, MRSL, MRS-LA, MRSL-LA, *L. plantarum* B2028 CFS, CFSL at pH 3.8 (Figure 5.3).

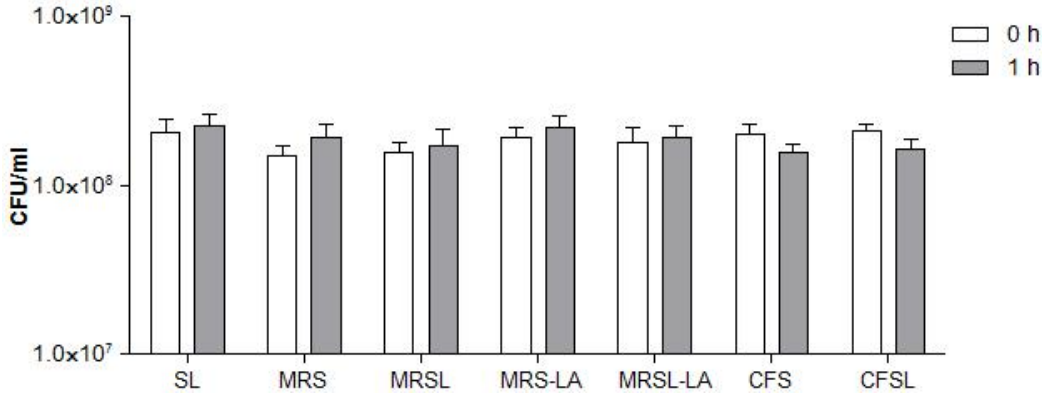


Figure 5.3 Numbers of viable STm SL1344nal^r cells before (0 hour, white bar) and after pre-incubation (1 hour, grey bar) in IPEC-J2 medium with *L. plantarum* B2028 supernatants (CFS, CFSL), lactic acid controls (MRS-LA, MRSL-LA) and pH controls (MRS, MRSL) (pH 3.8) at 10% (v/v) dilution factor. Non-buffered control (SL) was included and incubated only in IPEC-J2 medium. The assay was performed in duplicate on three separate occasions and the SEM is shown.

For the invasion assays the porcine jejunal epithelial cells (IPEC-J2) were used (Schierack *et al*., 2006) and assays were performed as previously described (Coconnier *et al*., 1997; Dibb-Fuller *et al*., 1999; Makras *et al*., 2006) (section 2.5.4) using the bacterial cells that were pre-incubated for 1 hour in the media preparations used for viability tests. The invasion of STm SL1344nal^r into IPEC-J2 monolayers (expressed as a % of the initial inoculum invaded) was significantly reduced after pre-incubation with *L. plantarum* B2028 supernatants (CFS, CFSL), lactic acid controls (MRS-LA, MRSL-LA) (P<0.001) and to a lesser extent by pH controls (MRS, MRSL) (P<0.05) (Figure 5.4). The impact of pH alone (MRS/MRSL) was not as extensive as either pH

and lactic acid (MRS-LA/MRSL-LA) and even lower to that of pH and additional components of the spent medium CFS/CFSL (P<0.01).

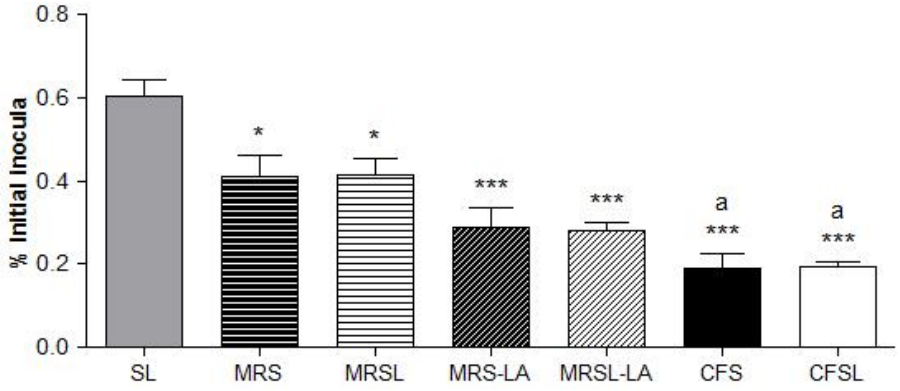


Figure 5.4 Invasion of STm SL1344nal^r to IPEC-J2 cells following pre-incubation with 10% (v/v) of *L. plantarum* B2028 supernatants CFS (black bar) and CFSL (white bar), lactic acid controls MRS-LA (hatched, black bar) and MRSL-LA (hatched, white bar) and pH controls MRS (horizontal lines, black bar) and MRSL (horizontal lines, white bar). Non-buffered control was included (SL, grey bar) and incubated only in IPEC-J2 medium. The assay was performed in duplicate on three separate occasions and the SEM is shown. Values significantly different from SL control are indicated by * P<0.05, *** P<0.001; values significantly different from the MRS and MRSL controls are indicated by the letter ^{a} P<0.01.

5.2.2 Reduction of *S.* Typhimurium adherence and invasion to IPEC-J2 cells using monolayer and three-dimensional model

Adhesion and invasion assays were performed as previously described (Dibb-Fuller *et al.*, 1999; Dibb-Fuller *et al.*, 2001; Searle *et al.*, 2009; Searle *et al.*, 2010) using different experimental conditions in a competition assay as listed in section 2.5.4. Both traditional IPEC-J2 monolayer and novel 3D cell models were utilised and the latter was based upon previous methods (Collins, 2010; Nickerson *et al.*, 2001; Searle *et al.*, 2010) (section 2.5.1.2). *L. plantarum* B2028 supernatants and MRS control (pH 3.8) were obtained as described in section 2.1.8 and used at a 10% (v/v) dilution factor.

A significant reduction in the number of STm SL1344 nal^r that had invaded was observed when monolayers were simultaneously incubated with *L. plantarum* B2028

and its CFSL or with CFSL on its own (P<0.001) as compared with the untreated STm SL1344 nal^r control (Figure 5.5). The reductions of invasion of STm SL1344 nal^r in monolayers were 20.6-fold and 10.0-fold respectively. Moreover, the adhesion of STm SL1344 nal^r to IPEC-J2 cells was also reduced when co-incubated with *L. plantarum* B2028 and its CFSL and CFSL devoid of bacterial cells (P<0.05). Interestingly, the probiotic without its supernatant or the prebiotic alone had no effect upon the adherence or invasion of STm SL1344 nal^r into porcine epithelial cells under the laboratory conditions used.

The results obtained using the 3D IPEC-J2 model were comparable to those acquired using the monolayer model (Figure 5.5), showing significant reduction in STm SL1344 nal^r adherence (P<0.05) to and invasion (P<0.001) into 3D IPEC-J2 cells when co-incubated with both cells and CFSL of *L. plantarum* B2028 or CFSL alone. *L. plantarum* B2028 and its supernatant reduced invasion of STm SL1344 nal^r 30.2-fold, whereas 13.0-fold reduction was observed when the cell free supernatant was administered solely.

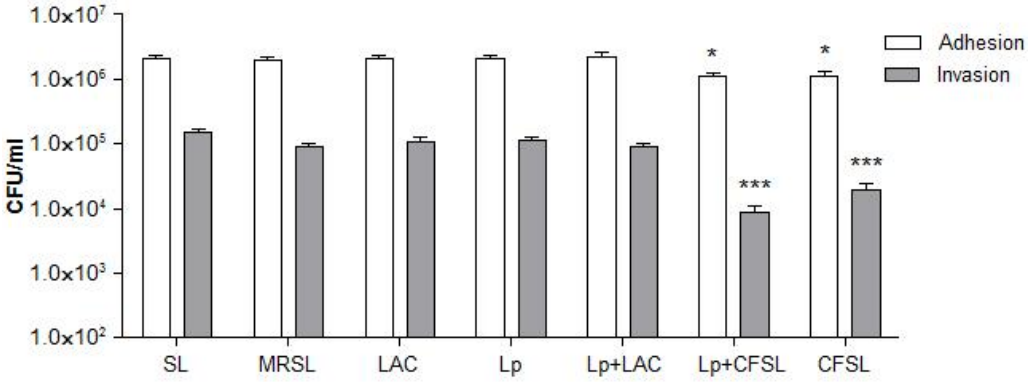


Figure 5.5 Adherence (white bars) and invasion (grey bars) of STm SL1344nal^r to IPEC-J2 monolayers in the presence of *L. plantarum* B2028 and LAC. All treatments containing *L. plantarum* B2028 CFSL and control MRSL (pH 3.8) were delivered at 10% (v/v) dilution factor, whereas LAC at 1% (w/v). The assay was performed in duplicate on three separate occasions and the SEM is shown. Values significantly different from the control (SL) are indicated by * P<0.05, *** P<0.001.

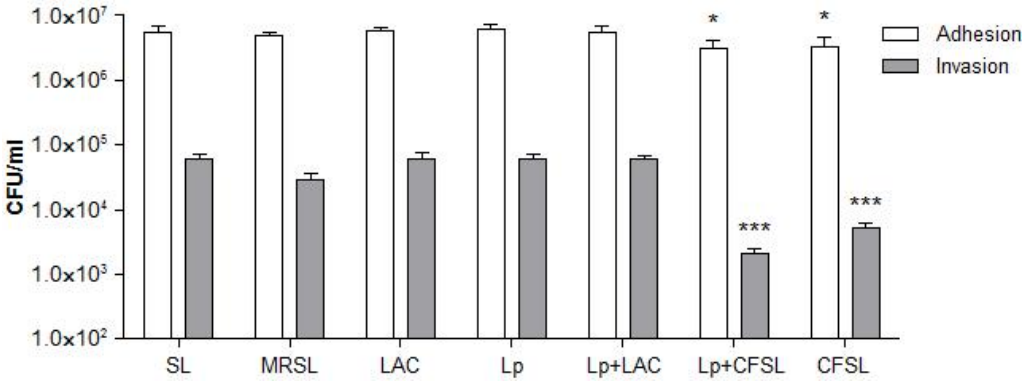


Figure 5.6 Adherence (white bars) and invasion (grey bars) of STm SL1344nal^r to IPEC-J2 3D cells in the presence of *L. plantarum* B2028 and LAC. All treatments containing *L. plantarum* B2028 CFSL and control MRSL (pH 3.8) were delivered at 10% (v/v) dilution factor, whereas LAC at 1% (w/v). The assay was performed in duplicate on three separate occasions and the SEM is shown. Values significantly different from the control (SL) are indicated by * $P<0.05$, *** $P<0.001$.

5.2.3 Porcine *in vitro* organ culture, the effect of *L. plantarum* B2028 and lactulose on *S*. Typhimurium association

A polarized *in vitro* organ culture (IVOC) model using CellCrown™ technology was employed as previously established by Collins *et al*. (2010) (section 2.5.6). Porcine intestinal explants from jejunum and colon were immobilized in CellCrowns™ (Scaffdex) with the mucosal side positioned upwards, placed into 24 well plates and submerged in a complete RPMI-1640 medium as previously described in section 2.5.6. Subsequently, the immobilised tissues were inoculated under the same experimental conditions that were used for adhesion and invasion assays (section 2.5.3) and incubated at 37°C in the presence of 5% CO_2.

Conversely to the results obtained from adhesion and invasion assays using monolayer and 3D cells, here the simultaneous co-incubation of STm SL1344nal^r with *L. plantarum* B2028 and CFSL (10% v/v) or CFSL (10% v/v) alone had no effect upon the association STm SL1344nal^r to porcine jejunal or colonic tissues.

Interestingly, a numerically small but statistically significant reduction in the number of STm SL1344nal^r associated with colonic tissue explants was observed when *L. plantarum* B2028 cells and LAC (1% w/v) were added simultaneously with STm SL1344nal^r ($P<0.05$) (Figure 5.7).

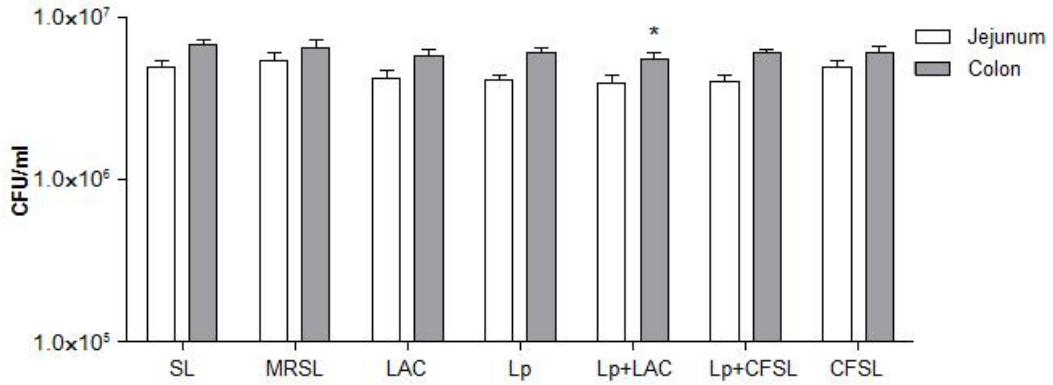


Figure 5.7 Association of STm SL1344nal^{r} to porcine jejunal (white bar) and colonic tissue explants (grey bar) in the presence of *L. plantarum* B2028 and LAC. All treatments containing *L. plantarum* B2028 CFSL and control MRSL (pH 3.8) were delivered at 10% (v/v) dilution factor, whereas LAC at 1% (w/v). The assay was performed in quadruplicate on two separate occasions and the SEM is shown. Values significantly different from the control (SL) are indicated by * $P<0.05$.

5.2.4 The effect of *L. plantarum* B2028 and lactulose on bacterial and organic acid changes in a porcine batch culture system infected with *S*. Typhimurium

5.2.4.1 Bacterial numbers changes

The experiment was set up and conducted as described in detail in section 2.1.14. Porcine faecal samples were checked for and showed the absence of *Salmonella* as described previously in section 2.6.5. The detection of the test organisms was by fluorescent *in situ* hybridization (FISH) (section 2.2.7). A total of four porcine batch culture fermentation systems were set up (section 2.1.14) and each was inoculated with an overnight culture of STm SL1344nal^r.

The numbers of *Salmonella*, detected using Sal303 FISH probe, were calculated over the 24 hour period of incubation in the CTR (STm SL1344nal^r only) porcine batch culture fermentation system and showed a marginal decline that was not statistically significant. The trend for all treatment groups was similar, but there were a few noticeable differences in LAC and *L. plantarum* B2028 and LAC (Lp + LAC) treatment groups. In the LAC treated porcine fermentation systems, *Salmonella* numbers increased significantly (P<0.05) in the 24 hour sample compared with the CTR 24 hour sample. In the Lp + LAC treated porcine fermentation systems, however, the numbers of *Salmonella* detected was significantly lower (P=0.05) in both the 10 and 24 hour samples compared with the 0 hour sample. Nevertheless, no significant differences were observed for Lp + LAC compared with CTR (Figure 5.8). No significant differences were observed for *L. plantarum* B2028 (Lp) treated porcine fermentation systems.

The numbers of *Lactobacillus-Enterococcus* detected using the Lab158 FISH probe over the 24 hour period of incubation were significantly increased with the inclusion of LAC and Lp + LAC to porcine fermentation systems (P<0.05). Interestingly, a particularly prominent increase in *Lactobacillus-Enterococcus* numbers was observed in the LAC treated porcine batch culture system after 10 hours of incubation (Figure 5.9). Mean values for *Lactobacillus-Enterococcus* calculated from all collected time points were significantly higher for Lp + LAC compared with CTR (P<0.05). No significant differences in *Lactobacillus-Enterococcus* were found in CTR or Lp treated fermentation systems.

No significant changes in bifidobacteria numbers with the inclusion of LAC, Lp + LAC or Lp were observed (P=1.00). Similarly, no significant changes were recorded in total numbers of bacteria numbers detected with DAPI stain (P=0.619).

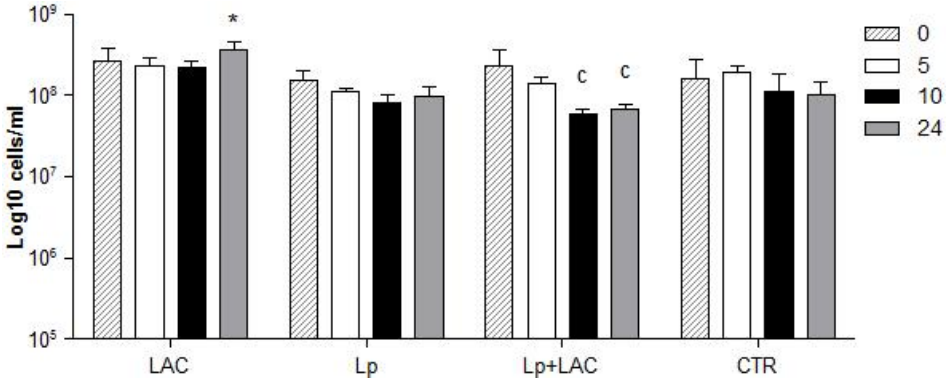


Figure 5.8 Bacterial numbers (Log_{10} cells/ml) enumerated using a Sal303 FISH probe over a 24 hour incubation period within porcine batch culture system inoculated with STm SL1344nal^r and treated with LAC, *L. plantarum* B2028 (Lp), *L. plantarum* B2028 + LAC (Lp + LAC), respectively. CTR was inoculated only with 10% (v/v) faecal slurry and STm SL1344nal^r. Bacterial numbers are showed at specific time points: 0 hour (hatched bar), 5 hours (white bar), 10 hours (black bar), 24 hours (grey bar). The assay was performed in duplicate on two separate occasions and the SEM is shown. Values significantly different from the CTR at the specific time point are indicated by * P<0.05 and * P<0.05; values significantly different from the 0 hour at 24 hours are indicated $^c P$<0.05.

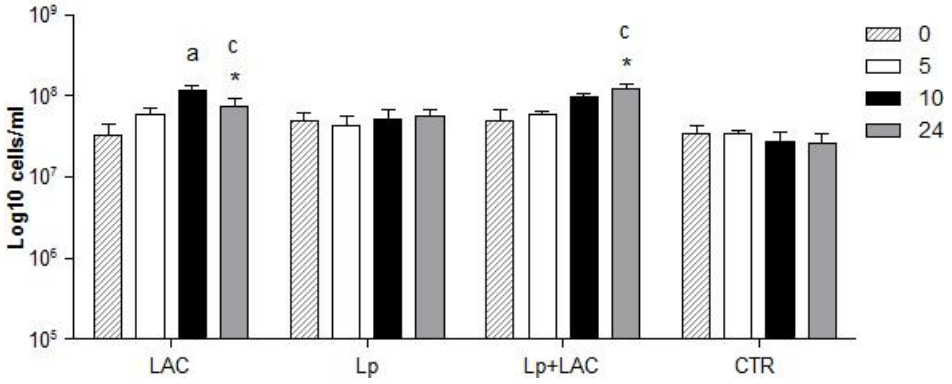


Figure 5.9 Bacterial numbers (Log_{10} cells/ml) enumerated using a Lab158 FISH probe over a 24 hour incubation period within porcine batch culture system inoculated with STm SL1344nal^r and treated with LAC, *L. plantarum* B2028 (Lp), *L. plantarum* B2028 + LAC (Lp + LAC), respectively. CTR was inoculated with only with 10% (v/v) faecal slurry and STm SL1344nal^r. Bacterial numbers are showed at specific time points: 0 hour (hatched bar), 5 hours (white bar), 10 hours (black bar), 24 hours (grey bar). The assay was performed in duplicate on two separate occasions and the SEM is shown. Values significantly different from the CTR at the 24 hours are indicated by * $P<0.05$; values significantly different from the 0 hour at 24 hours are indicated by $^aP<0.001$ and $^cP<0.05$.

5.2.4.2 Short chain fatty acids analysis

The production of short chain fatty acids (SCFA) in the porcine batch culture fermentation system (section 5.2.4.1) upon inclusion of the various treatment regimes was analysed by HPLC (section 2.4.1).

The addition of the prospective pre, pro or synbiotic was not correlated with the detectable changes in lactic acid concentrations (Figure 5.10). The CTR samples had reducing amounts of lactic acid so that by 24 hours the concentration was at the limits of detection. As predicted, by 24 hours, increases in SCFA concentrations (sum of acetic, propionic and butyric acids) were observed in all treatment groups and the control ($P<0.001$ and $P<0.05$ respectively) (Figure 5.11).

Delving deeper into the specific changes seen in the four treatments, after 5 hour incubation the total SCFA produced had risen in each of the four treatments about three-fold. However, there were no significant differences in SCFA concentration between treatments. After 10 hours, the general trend for increased accumulation of SCFA in all treatments continued to be observed but inclusion of LAC with or without

L. plantarum resulted in the significantly higher concentrations of total SCFA ($P<0.01$). After 24 hours, the increase in concentrations of total SCFA in all treatments was again observed. However, the amounts for treatments containing LAC (LAC and Lp + LAC) and for Lp alone were all significantly higher than CTR ($P<0.001$, $P<0.001$ and $P<0.05$, respectively).

Whilst Figure 5.11 shows the total SCFA data, more detailed analysis of the individual components of the SCFA was also undertaken and these data are shown in Figure 5.12. A general trend for the ratios of four SCFAs was shown across all treatment groups with concentrations of each being in the order of acetic > propionic > butyric > lactic acid. After 5 hour incubation there were no significant differences between treatments. However, after 10 hours for the Lp and CTR propionate concentrations were significantly less than all other treatments ($P<0.05$ and $P<0.001$). At the same time point acetate concentrations were significantly higher than butyrate. After 24 hour incubation, the concentrations of all acids in all treatments were higher than CTR. Also at this time point, acetic acid was higher versus those of butyrate for LAC and Lp ($P<0.01$ and $P<0.05$ respectively). In both LAC and Lp + LAC treatments butyrate concentrations were significantly higher than CTR ($P<0.001$).

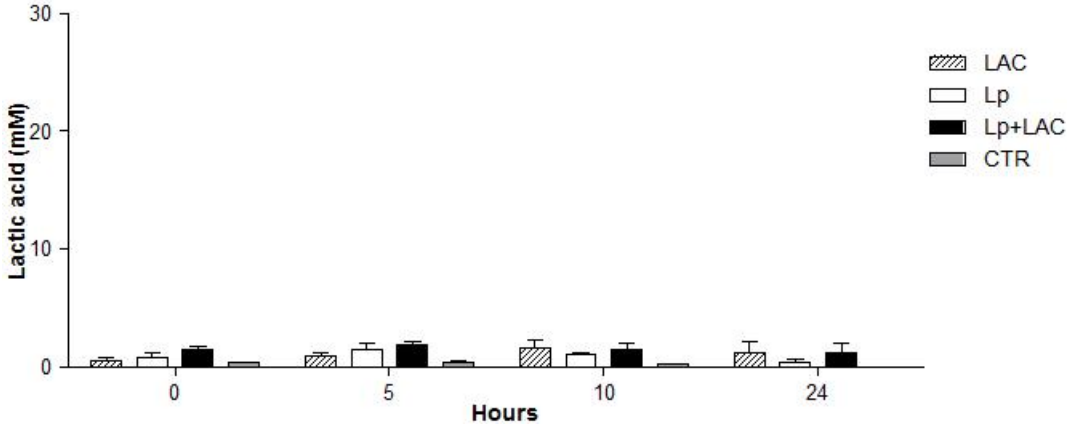


Figure 5.10 Concentrations (mM) of lactic acid at 0, 5, 10 and 24 hours of fermentation period, in a porcine batch culture system inoculated with STm SL1344nal^{r} and treated with LAC (hatched bar), *L. plantarum* B2028 (Lp) (white bar), *L. plantarum* B2028 + LAC (Lp + LAC) (black bar). CTR (grey bar) was included and inoculated with with 10% (v/v) faecal slurry and STm SL1344nal^{r}. The assay was performed in duplicate on two separate occasions and the SEM is shown.

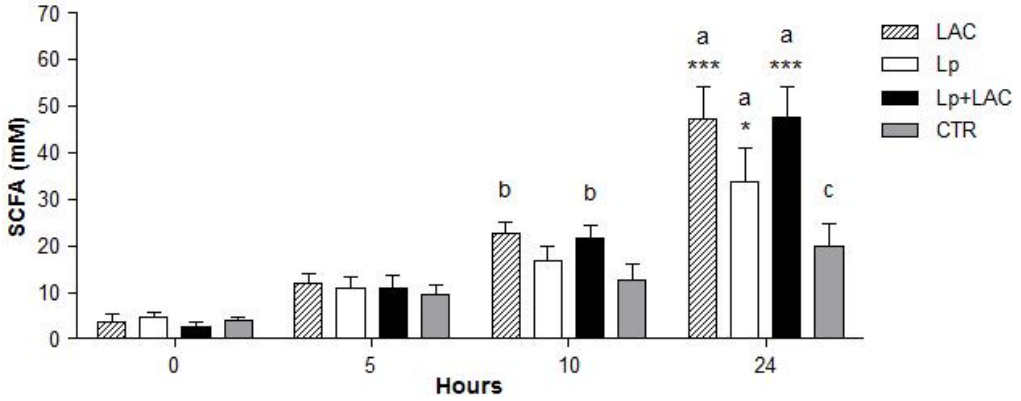


Figure 5.11 Concentrations (mM) of SCFA (sum of acetic, propionic and butyric acid) at 0, 5, 10 and 24 hours of fermentation period, in a porcine batch culture system inoculated with STm SL1344nal^{r} and treated with with LAC (hatched bar), *L. plantarum* B2028 (Lp) (white bar), *L. plantarum* B2028 + LAC (Lp + LAC) (black bar). CTR (grey bar) was inoculated only with 10% (v/v) faecal slurry and STm SL1344nal^{r}. The assay was performed in duplicate on two separate occasions and the SEM is shown. Values significantly different from the CTR at the specific time point are indicated by * P<0.05 and *** P<0.001; values significantly different from the 0 hour at 24 hours are indicated by ^{a}P<0.01, ^{b}P<0.01 and ^{c}P<0.05.

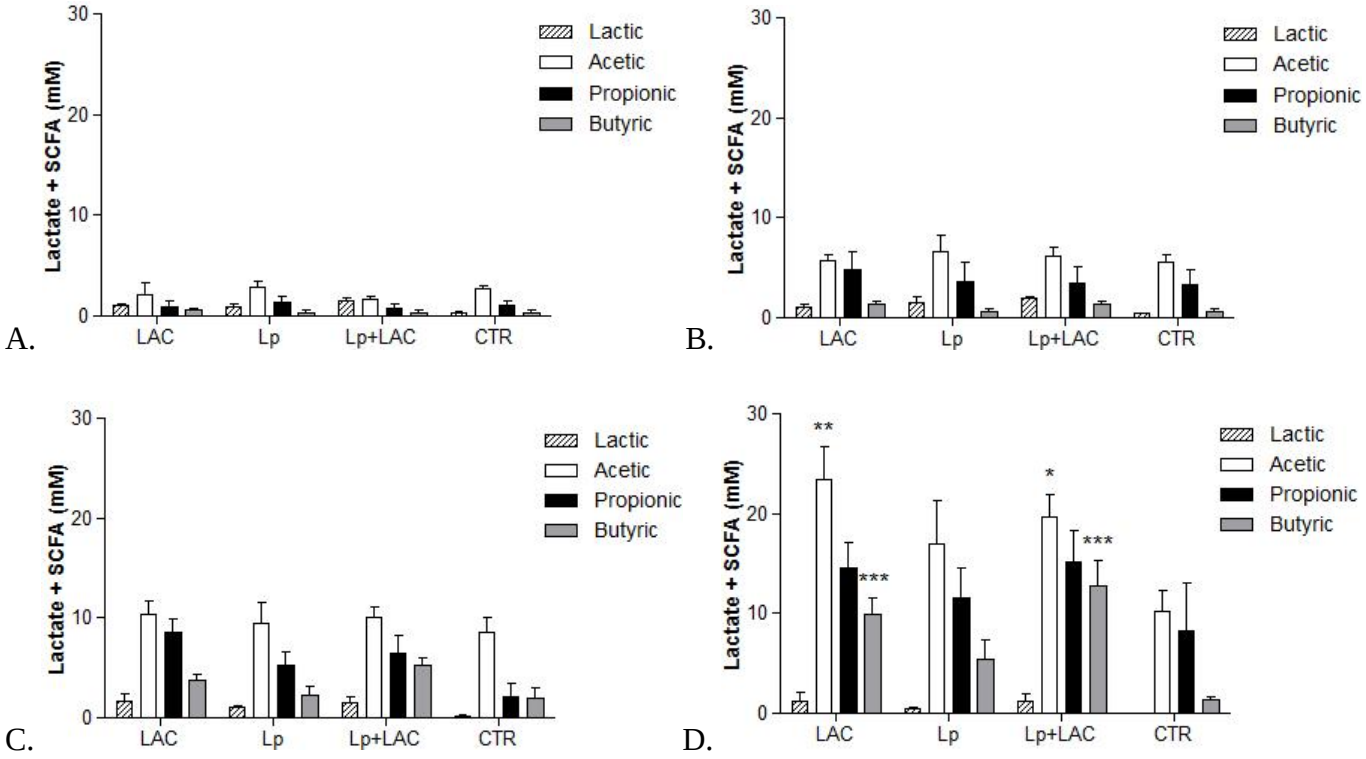


Figure 5.12 Concentrations (mM) of lactic (hatched bar), acetic (white bar), propionic (black bar) and butyric (grey bar) acid at times 0 hour (A), 5 hours (B), 10 hours (C), 24 hours (D) hours in a porcine batch cultures inoculated with STm SL1344nal^{r} and treated with LAC, *L. plantarum* B2028 (Lp), *L. plantarum* B2028 + LAC (Lp + LAC). CTR was inoculated only with 10% faecal slurry and STm SL1344nal^{r} only. The assay was performed in duplicate on two separate occasions and the SEM is shown. Values significantly different from the CTR at 24 hours are indicated by * P<0.05, ** P<0.01 and *** P<0.001.

5.3 Discussion

Studies presented in this chapter aimed to evaluate the anti-*Salmonella* activity of the synbiotic containing *L. plantarum* B2028 and LAC. The mechanisms of the antibacterial activity of probiotics are not yet fully defined, however, they seem to be multi-factorial as discussed earlier in this thesis (Servin, 2004). Therefore, a range of porcine *in vitro* models was employed to investigate the impact of the synbiotic and its constituent components upon the biology of STm SL1344 nal^r. Specifically, the various models assessed growth, adhesion/invasion to epithelial cells and survival in dynamic and complex batch culture system. Moreover, the studies in this section were performed with the view that use of adequate *in vitro* models is a precondition for a successful animal studies and essential from animal welfare perspective (Boyen *et al*., 2009; Russell and Burch, 1992).

Following from the previous findings where the growth of STm SL1344 nal^r was significantly inhibited by *L. plantarum* B2028 CFS, a pH-dependent antibacterial effect against the growth of STm SL1344 nal^r was demonstrated here. The growth of STm SL1344 nal^r with *L. plantarum* B2028 supernatants either non-buffered (pH3.8) or adjusted (pH 4.5) was significantly reduced compared with the growth in LB-G medium. Neutralisation of the *L. plantarum* B2028 cell free supernatants to pH 7.2 completely eliminated their antimicrobial effect on STm SL1344 nal^r. The pH-dependent mechanism of the antagonistic activity of *L. casei* GG on *Salmonella* was previously reported (Hudault *et al*., 1997), and this is in agreement with our findings. Nevertheless, it is important to remember that in the studies presented in this thesis supernatants were included at the 10% (v/v) dilution factor. So, whilst pH seems to be the primary effector, it may be argued that pH in combination with specific metabolites are important in inhibition of STm SL1344 nal^r growth. This concept is partially borne out by the findings in which we observed that lactic acid control (MRS-LA) at pH 3.8, had a similar inhibitory activity to the supernatants of *L. plantarum* B2028. Adjusting to pH 4.5, in contrast to *L. plantarum* B2028 supernatants (CFS, CFSL) the pH controls (MRS, MRSL) and one of the lactic acid controls (MRS-LA) resulted in loss of that activity against STm SL1344 nal^r.

In the later study where the STm SL1344 nal^r was pre-incubated with the *L. plantarum* B2028 supernatants or respective pH/lactic acid controls it was also observed that only CFS/CFSL had the significant inhibitory effect on STm SL1344

nal^r invasion when compared to the pH controls. These sets of data illustrate the importance of lactic acid in the anti-*Salmonella* activity of *L. plantarum* B2028 supernatant; yet it also shows that it is not exclusive factor of. Those results also fit previously published findings of both lactic acid and low pH playing an important role in the antibacterial activity of probiotics (De Keersmaecker *et al*., 2006; Makras *et al*., 2006; Servin, 2004). The organic acids and pH can affect microbes independently, yet as the pH determines the ratio of dissociated and non-dissociated forms of an acid near its pK_a it influence the effect of it (Eklund, 1983). This could explain gradual decrease in the effect of *L. plantarum* B2028 supernatant and lactic acid control on STm SL1344 nal^r growth. Although, both dissociated and non-dissociated forms of acids have been assigned for inhibitory effect towards various pathogens, nevertheless the latter was found to be more profound (Eklund, 1983, 1985). As the porcine luminal colonic content batch culture models showed, acetate was the most abundant organic produced in all of the treatments. Whilst the studies here focused on lactate, perhaps further studies with acetate and for that matter propionate and butyrate need to be performed to assess the relative contributions each SCFA makes to the inhibition of STm SL1344 nal^r. Certainly, acetate has been shown to be more inhibitory than lactate in auxanography experiments performed with *E. coli* (Woodward, personal communication). The data suggest that *L. plantarum* B2028 is heterofermentative producing equimolar amounts of lactic and acetic acids. It is not unreasonable to assume both lactic and acetic acids are inhibitory at the lower pHs tested here. Following on from this, inhibitory compounds other than lactic acid in lactobacilli supernatants have been reported (Fayol-Messaoudi *et al*., 2005; Silva *et al*., 1987). Interestingly Niku-Paavola *et al*. (1999) showed the presence of a low molecular mass compound in the supernatant of *L. plantarum* that was inhibitory towards the Gram-negative bacterium *P. agglomerans* in co-operation with lactic acid. Indeed, Alakomi *et al*. (2000) demonstrated the role of lactic acid as a permeabiliser of the Gram-negative bacteria outer membrane (OM), which could facilitate effective penetration of the numerous antimicrobial metabolites produced by the LAB that are normally unable to penetrate the OM or sensitize the gram negative bacteria to bacteriocins. It could possibly be hypothesised that the antimicrobial mechanism of *L. plantarum* B2028 supernatants against STm SL1344 nal^r is due to an as yet unknown metabolite that works synergistically with the lactic acid. In addition, and as suggested above with regard to

acetic acid, the presence and/or unique ratio of the various organic acids in the supernatant in conjunction with the pH may work synergistically to inhibit STm SL1344 nal^r. Further in depth studies targeting the presence of other compounds are necessary.

In order to initiate the infection process pathogenic bacteria must first adhere to the surface of the epithelial cells (Weinstein *et al*., 1998). Several studies indicated that LAB could prevent pathogens adhesion by competing for the same receptor sites, steric hindrance, co-aggregation or the production of antimicrobial molecules, thus resulting in reduced colonisation (Bernet-Camard *et al*., 1997; Bernet *et al*., 1994; Gueimonde *et al*., 2005; Lee *et al*., 2003; Mappley *et al*., 2011; Neeser *et al*., 2000). In the previous chapter, it was demonstrated that *L. plantarum* B2028 adhered to the IPEC-J2 cells in high numbers. In the adhesion and invasion assays using monolayer and 3D cells, a significant reduction of adhesion and invasion of STm SL1344 nal^r was observed when co-administered with *L. plantarum* B2028 cells with the supernatant or the supernatant alone. Neither MRS nor *L. plantarum* B2028 cells alone showed any effect upon the adherence or invasion of STm SL1344 nal^r. This suggests that low pH of the supernatant is at least partially responsible for reduction of *Salmonella* adherence and invasion into epithelial cells. This does not preclude the possibility that the inhibitory effects both on the growth and the adhesion/invasion of STm SL1344 nal^r could be partially attributable to a secreted unidentified antimicrobial substance. The antimicrobial effect of *L. acidophilus* LB CFS, attributable to the presence of a non-lactic acid molecule (s) was previously reported (Coconnier *et al*., 1997). Authors observed that pre-treatment of *S.* Typhimurium SL1344 with *L. acidophilus* LB CFS for 1 hour resulted in its significant decrease in association and even greater reduction of pathogen inhibition to Caco-2 cells. Those findings corroborate the results in this chapter, indicating that the *Salmonella* invasive phenotype was affected by the pre-treatment with *L. plantarum* B2028 supernatant and while lactic acid played important role, CFS/CFSL was even more potent. Interestingly, recent studies shed more light on more possible mechanisms of lactobacilli anti-*Salmonella* activity as it was demonstrated that pre-treatment of *S.* Typhimurium SL1344 with the CFS of from *L. acidophilus* LB caused temporary loss swimming motility without effecting flagella expression, that in turn resulted in delayed entry into Caco-2/TC7 cells (Lievin-Le Moal *et al*., 2011).

It is possible to exclude a co-aggregative mechanism of action of *L. plantarum* B2028 which has been observed for *L. reuteri* LM1 strain (Mappley *et al*., 2011) because washed *L. plantarum* B2028 cells showed no effect upon reduction of the adhesion or invasion of STm SL1344 nal^r. Interestingly, Bernet *et al*. (1994) demonstrated that when the spent culture supernatant of *L. acidophilus* LA1 was discarded and replaced with the fresh culture medium a significant loss of adhesion to intestinal cells of this strain occurred. The *Lactobacillus* spp. adherence process to epithelial cells is a complex mechanism which seems to be influenced by multiple factors like bacterial physiology and physicochemical parameters but also the pH (Greene and Klaenhammer, 1994; Henriksson *et al*., 1991; Pelletier *et al*., 1997). These findings collectively suggest an explanation why *L. plantarum* B2028 cells devoid of the supernatant showed no effect against *Salmonella* adherence/invasion.

CFSL alone reduced STm SL1344 nal^r invasion by 10.0-fold and *L. plantarum* with CFSL by 20.6-fold in monolayers and 13.0-fold, 30.2-fold in 3D cells respectively. Although, this reduction was very significant with both treatments when the probiotic was administered together with its supernatant this effect was greatest. It might be, that regardless of pH and lactic acid activity other components of CFSL enhance *L. plantarum* B2028 adherence to epithelial cells perhaps promoting stearic hindrance for the attachment of STm SL1344 nal^r. The importance of lipoteichoic acid (LTA) and expolysaccharide (EPS) molecules in the mechanism of lactobacilli adhesion to epithelial cells has been demonstrated previously (Granato *et al*., 1999; Lebeer *et al*., 2008; Ruas-Madiedo *et al*., 2006; Sherman and Savage, 1986). The impact of pH or other metabolites in the supernatant on enhancing the role of these molecules in adherence and/or stearic hindrance needs to be further assessed.

Of all the studies performed in this chapter it may be argued that IVOC was the most physiologically relevant because the system uses entire tissue with an integrated structure as found *in vivo*. IVOC model was formerly used to study the adhesive properties of pathogens and the associated host cellular responses (Girard *et al*., 2007; Phillips and Frankel, 2000). Furthermore, Henriksson *et al*. (1991) employed the porcine gastric squamous epithelium IVOC model to study the adhesion characteristics of *L. fermentum*. However, a limitation of this model in our study was the inability to differentiate STm SL1344 nal^r bacteria that had adhered or invaded; quantification was, therefore, of the total associated STm SL1344 nal^r. Unlike the

monolayer and 3D cell model data, no significant differences were observed upon administration of *L. plantarum* B2028 with CFSL or CFSL alone in the number of associated STm SL1344 nal^r. This result does not rule out the possibility that, although STm SL1344 nal^r associated, invasion has been inhibited. In IVOC the only significant reduction of association was with the Lp + LAC treatment (P<0.05). Neither Lp nor LAC alone reduced association indicating that a only the combined treatment was effective in inhibiting association of STm SL1344 nal^r with tissue. Perhaps a combination of stearic hindrance by bound Lp, co-aggregation with Lp and blocking of binding sites by the oligosaccharide LAC collectively had the effect. None of these options are supported by other data from this work, however, although Searle *et al*. (2009, 2010) did show that GOS acted as a barrier to adherence in their *in vivo* studies. In hind-sight it would have been useful to have undertook cellular response studies in the IVOC studies as these may have revealed greater detail relating to host cell interactions.

The studies described in section 3.2.5 of Chapter 3 demonstrated significant increases of *Lactobacillus-Enterococcus* and SCFA concentrations with the inclusion of LAC, FOS and their synbiotic combination with *L. plantarum* B2028 into a porcine batch culture fermentation system. The enhancement of LAB numbers and SCFA concentrations have been previously linked with the reduction of *Salmonella* (Meynell, 1963; Prohaszka *et al*., 1990). Therefore, the effect of the synbiotic combination of *L. plantarum* B2028 and LAC upon *Salmonella*, bifidobacteria and *Lactobacillus-Enterococcus* numbers and the end product metabolites was evaluated using a porcine colonic luminal content batch culture model. As demonstrated, the inclusion of LAC alone significantly increased STm SL1344nal^r numbers at 24 hours as compared with CTR (P<0.05). This is perhaps a surprising result as the expectation might have been that LAC would be metabolised by bifidobacteria and lactobacilli to produce inhibitory substances. Interestingly, there was a significant increase in the numbers of organisms binding the Lab158 probe, which was observed after 10 and 24 hours compared with the 0 hour time point (P<0.001; P<0.05 respectively). As previously demonstrated, STm SL1344nal^r is unable to utilise LAC as a sole carbon source. Taken collectively, these data suggest that the increase in the numbers of STm SL1344nal^r may reflect its growth on end products or by-products of the LAC fermentation by other members of porcine colonic microbiota. The data also suggest that those intermediates were readily bio-available and not in an

environment which was inhibitory. These finding are similar to those of Petersen *et al*. (2009) and support the previous studies of Martin-Pealez *et al*. (2010), who demonstrated no reduction in *Salmonella* numbers in porcines with the administration of lactulose in their diets.

Interestingly, a significant decrease in STm SL1344nal^r numbers was observed with the prospective synbiotic after 10 and 24 hours of incubation compared with 0 hour (P<0.05). There was a concomitant gradual increase in *Lactobacillus-Enterococcus* numbers from 0 to 24 hours (P<0.05). The inclusion of either of pre, pro or synbiotic was not correlated with increased lactic acid concentrations, which was previously observed in the first batch culture study. As mentioned previously, it is most likely that other bacterial species present in the porcine faecal slurry had utilised the lactate which in turn added to the pool of total SCFAs (Duncan *et al*., 2004; Hashizume *et al*., 2003; Ushida *et al*., 2002). The significant increase in SCFA concentrations (sum of acetic, propionic and butyric acids) from 0 to 24 hour time point was observed for all treated vessels (P<0.001) including CTR (P<0.05). The fact that fermentation in the control also yielded these acids indicates that there was fermentable substrate in the control vessel to yield these products. Addition of LAC and Lp + LAC enhanced yields most significantly compared with CTR (P<0.001). Moreover where LAC and Lp + LAC were included the concentrations of butyrate were significantly higher than CTR (P<0.001). The results showed that inclusion of both LAC alone or the synbiotic containing *L. plantarum* B2028 and LAC resulted in the increased numbers of those bacteria that bound the Lab158 probe and SCFA concentrations. Importantly, it was only with the synbiotic that the numbers of bacteria that bound the Sal303 probe decreased. This might indicate that active metabolites other than SCFA could have played role in reducing STm SL1344nal^r survival. Further to described above studies, it was demonstrated by Coconnier-Polter *et al*. (2005) that *L. acidophilus* LB CFS exerted remarkable *S*. Typhimurium SL1344 killing activity after 4 hour exposure to CFS, attributable to non-lactic acid molecule(s), more precisely CFS promoted depletion of intracellular ATP, lipopolysaccharide release, sensitizes *Salmonella* membrane and increases its permeabilization. It could possibly be hypothesised that this could also be true in case of this complex microbial environment.

Moreover, by additionally introducing the *L. plantarum* B2028 we might have created an unfavorable niche for STm SL1344nal^r. Due to the character of this model

(static closed fermentation system) it is not possible to observe other effects which this complex environment may play on STm SL1344nal^r colonisation. For instance, various studies showed that different SCFA can regulate the invasive phenotype of *Salmonella* by up or down-regulating virulence gene expression (Lawhon *et al*., 2002; Van Immerseel *et al*., 2004a; Van Immerseel *et al*., 2004b). Butyrate and propionate in particular have been reported to reduce the expression of the invasion genes (Gantois *et al*., 2006; Lawhon *et al*., 2002; Van Immerseel *et al*., 2004b). In both batch culture studies inclusion of LAC and *L. plantarum* B2028 + LAC resulted in higher butyrate concentrations than CTR, which has been demonstrated to be associated with reduced faecal shedding and intestinal colonisation in pigs (Boyen *et al*., 2008). In addition, organic acids might play a positive role in digestion, absorption and contribute to epithelial cell proliferation in pigs (Mroz *et al*., 2005).

Collectively, the findings presented here show complexity of the *L. plantarum* B2028 activity against STm SL1344nal^r, and unquestionably further investigation is required to evaluate further mechanisms underlying that activity. However, those results encourage further assessment of *L. plantarum* B2028 and LAC in an animal model as the next step of the selection for a successful synbiotic combination, which could be incorporated into a feed to control zoonotic pathogens like *S.* Typhimurium.

Chapter 6

Evaluation of an in feed administration of *L. plantarum* B2028 and lactulose to pigs experimentally challenged with *S.* Typhimurium – an *in vivo* pilot study

6.1 Introduction

Despite the continuing decline in the number of laboratory-confirmed human salmonellosis cases in the UK, a total of 10,071 cases were confirmed in 2009 and 9,685 cases in 2010 (DEFRA, 2009, 2010). *S.* Typhimurium remained the second most commonly reported serovar in those cases during this time frame and over those two years increased by 4.6% (DEFRA, 2010).

Pig infections caused by *S.* Typhimurium result in significant economic losses and pose an important human health issue (Boyen *et al*., 2008; Meyerholz *et al*., 2002) with pork and pork products considered as one of the major sources of human salmonellosis (Lo Fo Wong *et al*., 2002). In pigs, non-typhoidal salmonellosis is clinically manifested by onset of diarrhoea, pyrexia, in-appetence and lethargy. As disease is generally characterised by low mortality, but high morbidity rates, the majority of the pigs recover from infection and often become asymptomatic carriers (Wilcock and Olander, 1977). Those carrier pigs act as a reservoir and result in long term persistence of *Salmonella* in herds and thereby sustain risk of food chain contamination (Boyen *et al*., 2008).

For many years antimicrobial growth promoters have been used in livestock feed due to their beneficial effects on feed conversion rates and reduced disease incidence (Delsol *et al*., 2004; McEwen and Fedorka-Cray, 2002). Nevertheless, prolonged use of antimicrobial agents in veterinary medicine and animal husbandry ultimately resulted in the development of resistance in pathogenic bacteria (Aarestrup and Carstensen, 1998; Boerlin *et al*., 2001; Taylor, 1997). Subsequently, due to the

human health concerns and follow-on consequences manifested by increased frequency of treatment failures and increased severity of infections (FAO, WHO 2004), the EU banned the use of antimicrobial growth promoters in livestock as from 2006 (Castanon, 2007). Following termination of the prophylactic use of in-feed antibiotics, there resulted an increased need for research towards growth improvement and alternative control strategies (Castillo *et al*., 2008; McEwen and Fedorka-Cray, 2002) (FAO, WHO 2004), which would offer comparable beneficial effects and stimulate production.

Various alternative in-feed approaches have been studied to date and some are used commercially with, for example, organic acids, herbal extracts, probiotics, prebiotics and synbiotics as feed additives (Collado *et al*., 2007; Collins *et al*., 2009; Mappley *et al*., 2011; Partanen *et al*., 2006; Searle *et al*., 2009; Szabo *et al*., 2009), which were discussed in detail in Chapter 1 of this thesis. Supplementation of pig diet with various competitive exclusion cultures has been reported to reduce the severity of clinical signs and lower the numbers of *Salmonella* in the faeces (Fedorka-Cray *et al*., 1999; Genovese *et al*., 2003). In addition, Casey *et al*. (2007) concluded that mixtures of probiotic strains improved both clinical and microbiological outcome of salmonellosis.

The results presented in the previous chapters of this thesis demonstrated that exposure of STm SL1344nal^r to *L. plantarum* B2028 + LAC supernatant significantly reduced growth and also invasion of STm SL1344nal^r into IPEC-J2 cells. Furthermore, addition of LAC and *L. plantarum* B2028 + LAC to porcine faecal batch cultures, resulted in increases in lactobacilli and decreases in STm SL1344nal^r within the system. *In vitro* studies are advantageous in that they generate data that, in this set of studies, indicate this synbiotic combination may be an effective control of *S.* Typhimurium infection in the pig before and without recourse to *in vivo* studies. However, *in vitro* studies cannot replace the *in vivo* model in its complexity (Boyen *et al*., 2009). Therefore, this last chapter describes a use of a porcine *in vivo* model to test the efficacy of LAC and *L. plantarum* B2028 alone and as a synbiotic combination to control infection caused by STm SL1344nal^r.

6.2 Results

6.2.1 Experimental design summary

To study the individual and combined impact of *L. plantarum* B2028 and lactulose (LAC) in a pig model of *Salmonella* infection, the following study design was implemented (Figure 6.1, further details in sections 2.6.3 & 2.6.4). Briefly, twenty four 4-week-old commercial piglets were separated into four experimental groups and allowed 1 week to acclimatise (day -14 to -7) to the environment and diet. At 5 weeks of age (day -7 onwards), the diets for each of three test groups were supplemented with 1% (w/v) LAC (LAC group), *L. plantarum* B2028 (10^{10} cfu/animal/day) (Lp group), and combination of both (Lp + LAC group), and the treatments were provided until the end of the study (day -14 to 10). Piglets from control group (CTR group) continued to receive the original diet without supplements. Subsequently, 7 days after in-feed treatment inclusion, piglets were challenged with *S.* Typhimurium SL1344nal^r (STm SL1344 nal^r) (5×10^8 cfu) (day 0). The presence of *Salmonella* was evaluated before the challenge on four occasions (days -11, -7, -3, -1) and afterwards daily from day 0 to 10. The numbers of *Lactobacillus* spp. and *E. coli* in faecal samples from each of the piglets in all study groups were evaluated before and after *Salmonella* challenge, on days: -7, -1, 4, 7 and 10.

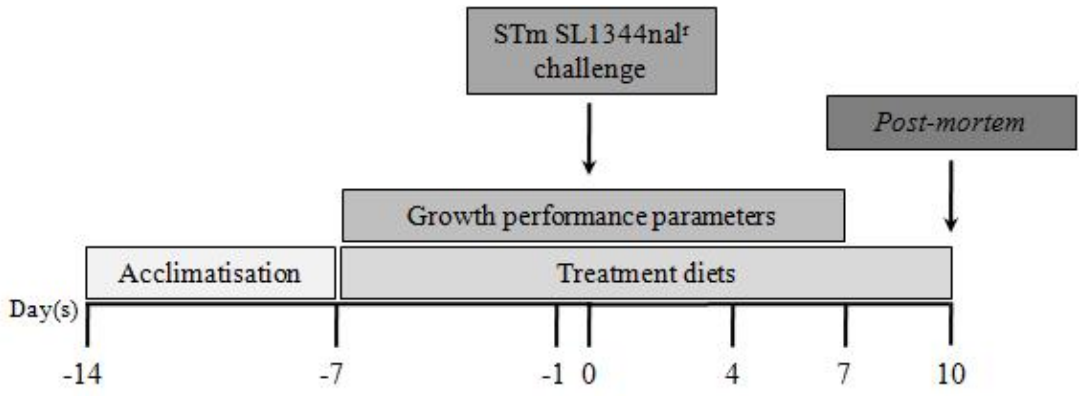


Figure 6.1 Graphic summary of an *in vivo* study design.

6.2.2 Physiological and zootechnical performance

Piglets in their respective experimental groups responded well to the incorporation of *L. plantarum* B2028 alone, LAC alone and *L. plantarum* B2028 + LAC together into a feed. As anticipated no signs of diarrhoea or decline in physiological performance were observed following inclusion of the feed additives before the challenge with STm SL1344nal^r (days -7 to 0).

Experimental challenge with STm SL1344nal^r resulted in the development of mild clinical signs of salmonellosis in all groups. Those, were limited to slight pyrexia, with the temperature 40°C or above in two piglets during the first 3 days in each experimental group and in further 2/6 piglets in the group treated with LAC and the un-treated control group from day 3 onwards. Figure 6.2, shows the mean rectal temperatures in each study group. There were no statistically significant differences between groups.

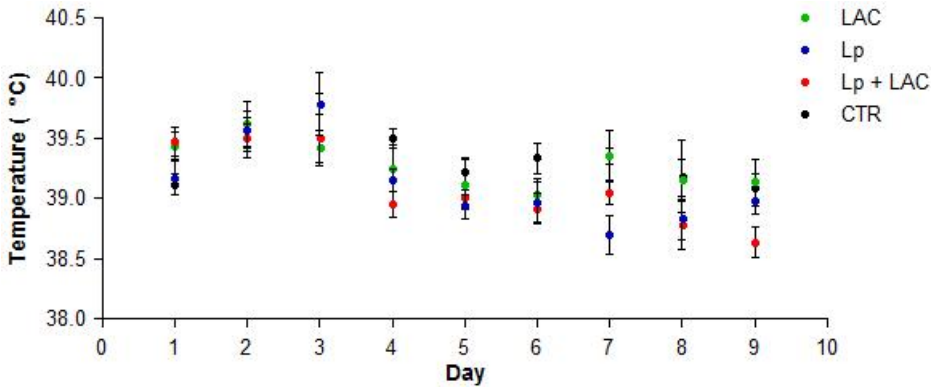


Figure 6.2 Rectal body temperature (˚C) of piglets experimentally challenged with STm SL1344nal^r and treated with LAC (green circles), *L. plantarum* B2028 (blue circles), *L. plantarum* B2028 + LAC (red circles) and fed un-treated diet (black circle). Animals were monitored daily until day 9 after challenge. Presented values are means of six animals in each of the experimental group and the SEM is shown.

Soft faeces were observed in the majority of piglets during the first four days after STm SL1344nal^r challenge and intermittently throughout the study, with no observable differences between the treatment groups.

The body weights of piglets, monitored weekly before and after *Salmonella* challenge (days -7 to 7), increased regularly and showed values comparable to those of normal development of body weight in piglets at that age. This increase in weight

from the beginning of the feeding trial was significant in animals from all four treatment groups ($P<0.001$) with no significant difference between them (Table 6.1).

Administration of LAC, *L. plantarum* B2028 and *L. plantarum* B2028 together with LAC had no significant effect on the average feed intake or the gain to feed ratio during the pre and post infection period as compared with un-treated control group (Table 6.1). However, it was interesting to note that the Lp + LAC group showed the greatest ADG during the *Salmonella* challenge compared with any other group at any other time.

Table 6.1 Pig growth performance parameters (weight, average daily gain, average daily feed intake and gain to feed ratio).

Parameter	**Experimental group**			
	LAC	***L .plantarum* B2028**	***L. plantarum* B2028 + LAC**	**Control**
Weight (kg)				
Day -7	11.10 ± 0.58	10.90 ± 0.31	10.73 ± 0.57	10.83 ± 0.63
Day 0	13.50 ± 0.64	13.70 ± 0.45	13.23 ± 0.73	13.43 ± 0.63
Day 7	16.23 ± 0.64	16.10 ± 0.46	16.40 ± 0.74	16.20 ± 0.62
Average daily gain (ADG) (g)				
Day -7 to 0	343 ± 35.3	400 ± 23.3	357 ± 43.4	371 ± 23.3
Day 0 to 7	390 ± 48.1	342 ± 51.1	452 ± 22.6	395 ± 20.0
Day -7 to 7	366 ± 27.0	371 ± 21.5	404 ± 25.7	383 ± 11.3
Average daily feed intake (ADFI) (g)				
Day -7 to 0	976 ± 39.4	930 ± 46.4	914 ± 42.4	954 ± 38.4
Day 0 to 7	1083 ± 0	1026 ± 37.6	1083 ± 0	1096 ± 9.91
Day -7 to 7	1029 ± 34.0	978 ± 44.7	998 ± 43.9	1011 ± 35.0
Gain to feed ratio (G:F)				
Day -7 to 0	0.35	0.43	0.39	0.38
Day 0 to 7	0.36	0.34	0.41	0.36
Day -7 to 7	0.35	0.37	0.40	0.37

6.2.3 Effect of feeding *L plantarum* B2028 and lactulose on *S*. Typhimurium faecal shedding

Isolation of STm SL1344nal^r from faeces was carried out by direct plating onto BGA supplemented with 15 µg/ml nalidixic acid and further enrichment as described previously in section 2.6.5. Throughout the post-infection period isolation of pathogen via direct plating was limited and only achieved for some of the animals within each experimental group and sporadically, which was most likely a result of the low numbers shed by some of animals rather than low sensitivity of the isolation method, especially as enrichment was used. Due to intermittent *Salmonella* shedding a scoring system was applied giving score values to each animal accordingly: (0) faeces negative for *Salmonella* on direct culture and following enrichment; (1) negative on direct culture but positive after enrichment; (2) positive on direct culture (and enrichment) with *Salmonella* counts up to 3.5 Log cfu/g faeces; (3) positive on direct culture (and enrichment) with counts from 3.5 Log cfu/g to 5 Log cfu/g faeces and (4) positive on direct culture (and enrichment) with counts above 5 Log cfu/g faeces.

Five of the six piglets in each experimental group were confirmed positive by enrichment at day 1 after challenge (Table 6.2). Only in the LAC group 6/6 animals were found *Salmonella* positive, this was at days 3 and 10 after challenge. Throughout the entire post-infection period 5/6 pigs from un-treated control group were *Salmonella* positive on five days after challenge, on four days from *L. plantarum* B2028 group, whereas only once from the synbiotic group. Moreover, only 3/6 animals treated with *L. plantarum* B2028 + LAC were found to be *Salmonella* positive over six days. The mean number of *Salmonella* shedding piglets throughout the entire 10 days in the group treated with *L. plantarum* B2028 + LAC was significantly reduced in comparison to the number of piglets in the group treated with *L. plantarum* B2028 alone ($P<0.05$) and a trend toward significance was observed in comparison to un-treated control and LAC treated groups ($P=0.06$).

Moreover, on average the shedding, as evaluated by score values and calculated for entire post challenge period for each experimental group, showed lower values for *L. plantarum* B2028 + LAC that were significantly different in comparison to un-treated control ($P<0.05$). The highest *Salmonella* score was detected on day 1 and 3 post challenge with the mean values of 2.6, 2.3, 2.1 and 2.8 for LAC, *L. plantarum* B2028, *L. plantarum* B2028 + LAC and un-treated group, respectively on day 1 post

challenge. STm SL1344nal^{r} was isolated from faeces by direct plating from 5/6 piglets in LAC and un-treated control group and from 4/6 piglets in groups treated with *L. plantarum* B2028 and *L. plantarum* B2028 + LAC. On day 3, the *Salmonella* score values were 2, 1.5, 1.33 and 2.1 for LAC, *L. plantarum* B2028, *L. plantarum* B2028 + LAC and control group respectively with 4/6 piglets (LAC and un-treated group) and 3/6 piglets (*L. plantarum* B2028, *L. plantarum* B2028 + LAC group) were positive on direct culture. A gradual decrease in the number of *Salmonella* positive faecal samples detected by direct culture and lower *Salmonella* score was observed after that point with mean score values no higher than 1.5 for all experimental groups except for day 7 for un-treated and day 10 for LAC group with 1.8 and 2 mean values, respectively (Figure 6.3). During the 10 days post infection the overall trend for *Salmonella* shedding calculated as the sum of score values was Lp + LAC (8.83) < Lp (11.0) < LAC (11.6) < CTR (12.8).

Table 6.2 The number of *Salmonella* positive faecal samples collected from piglets (*n*=6) in each experimental group during post-infection period (10 days).

Days	**Experimental group**			
	LAC	***L. plantarum* B2028**	***L. plantarum* B2028 + LAC**	**Control**
1	5/6	5/6	5/6	5/6
2	2/6	3/6	2/6	3/6
3	6/6	4/6	3/6	5/6
4	2/6	5/6	2/6	4/6
5	3/6	3/6	3/6	3/6
6	2/6	5/6	3/6	3/6
7	4/6	5/6	3/6	5/6
8	4/6	4/6	3/6	4/6
9	5/6	4/6	4/6	5/6
10	6/6	4/6	3/6	5/6

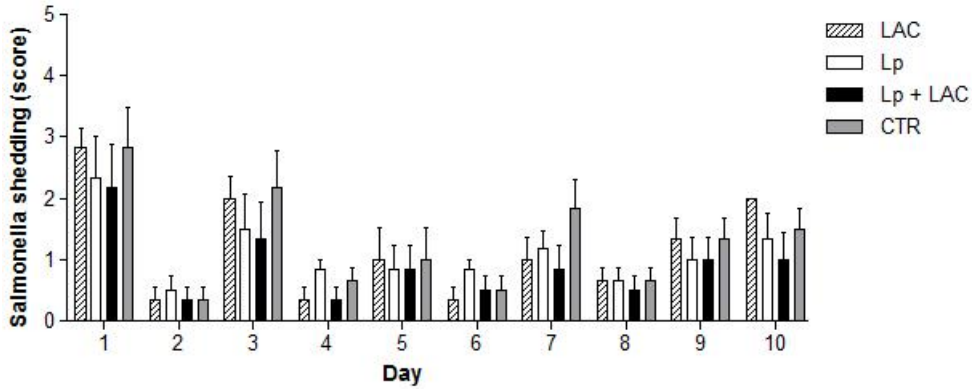


Figure 6.3 *Salmonella* faecal shedding results from the feeding trial evaluated during 10 days post STm SL1344nal^{r} challenge. Respective experimental groups consisted of six piglets and each was treated with LAC (hatched bar), *L. plantarum* B2028 (white bar), *L. plantarum* B2028 + LAC (black bar) and fed un-treated diet (grey bar). The scoring system was used for the data analysis, indicating the individual piglets with the lowest score value 0 were negative and with the highest value 4 shed more than 5 Log cfu/g faeces. Presented values (scores) are means of six animals in each of the experimental group and the SEM is shown.

6.2.4 *S*. Typhimurium dissemination in tissues collected at *post-mortem*

Animals shedding *Salmonella* throughout the study were selected and examined at *post-mortem* as described in section 2.6.6. *Salmonella* was isolated from jejunum, ileum, colon, caecum and mesenteric lymph nodes in all experimental groups. From jejunum, *Salmonella* was only isolated from animals in LAC and un-treated group. No *Salmonella* was recovered from liver and spleen of all animals subjected to *post-mortem* examination.

To compare the colonisation level between experimental groups the scoring system was applied as described in previous section and presented in Figure 6.4. No differences were found among the treatments.

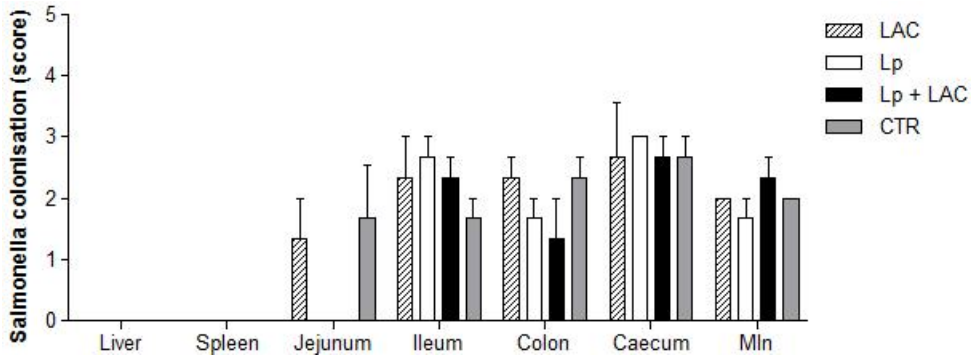


Figure 6.4 *Salmonella* colonisation in porcine tissues sampled at *post-mortem* at day 10 post STm SL1344nal^r challenge. Piglets within respective experimental group were treated with LAC (hatched bar), *L. plantarum* B2028 (white bar), *L. plantarum* B2028 + LAC (black bar) and fed un-treated diet (grey bar). Presented values (scores) are means of three animals in each of the experimental group and the SEM is shown.

6.2.5 Effect of feeding *L. plantarum* B2028 and lactulose on pig faecal microbiota

Lactobacilli and *E. coli* numbers from faecal samples were determined as described previously in section 2.6.5. To evaluate the outcome of the respective diets on those bacterial groups, faecal samples were collected and enumerated twice (days -7 and -1) before STm SL1344nal^r challenge and also on days 4, 7 and 10 after STm SL1344nal^r challenge (Figure 6.5 & 6.6).

6.2.5.1 *Lactobacillus* spp. changes

The inclusion of either LAC, *L. plantarum* B2028 or *L. plantarum* B2028 + LAC in the diet had no significant effect on the numbers of lactobacilli during the first week of diet administration (days -7 to -1). Following STm SL1344nal^r challenge (day 4), decreases in the numbers of lactobacilli in comparison to the pre-challenge values were observed in *L. plantarum* B2028 + LAC group (P=0.055). However, on day 7 after challenge the number of lactobacilli isolated from that group was again higher and significantly higher than in the un-treated control group (P<0.05). No significant differences in the numbers of lactobacilli in piglets treated with LAC, *L. plantarum* B2028, *L. plantarum* B2028 + LAC or un-treated control group were found on the last day of the study (day 10 after challenge). However the tendency to an increase in the number of lactobacilli in comparison to the control

group (P=0.059) was observed in the group treated with *L. plantarum* B2028 + LAC. Colonies from each plate taken for quantification were randomly selected for oxidase and catalase testing. All tested isolates were confirmed to be oxidase and catalase negative and therefore believed that all bacteria isolated on MRS medium were of *Lactobacillus* genus.

In addition, the presence of *L. plantarum* spp. in the faeces was examined prior to and after STm SL1344nal^r challenge using the previously described PCR (Kwon *et al*., 2004). Faeces of all piglets were negative for *L. plantarum* by PCR prior to feeding the experimental diet (day -7). Following diet inclusion (day -1), *L. plantarum* could be detected in animals from each of the experimental groups, however in those groups not receiving *L. plantarum* B2028 only one of six animals tested positive. The percentage of animals positive for *L. plantarum* by PCR was equal to or greater than 50% in the two groups receiving prospective probiotic or the synbiotic combination. Similar findings were made at day 4 and 10 after SL1344nal^r challenge (Table 6.4).

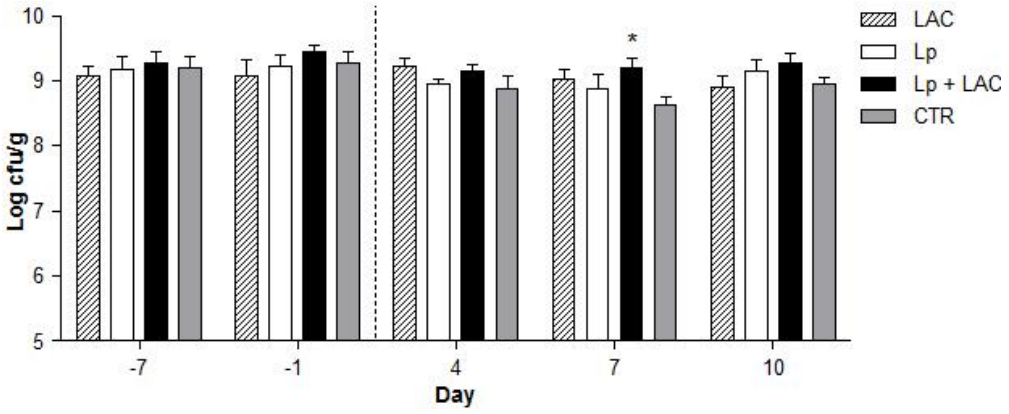


Figure 6.5 Counts of *Lactobacillus spp.* (Log cfu/g) recovered from faeces of piglets treated with LAC (hatched bar), *L. plantarum* B2028 (white bar), *L. plantarum* B2028 + LAC (black bar) and un-treated control group (grey bar) before and after STm SL1344nal^r challenge. Dotted line indicates STm SL1344nal^r challenge and day 0 of post-infection period. Presented values are means of six animals in each of the experimental group and the SEM is shown. Values significantly different from the control at the specific time point are indicated by * P<0.05.

Table 6.3 The number of positive samples for *L. plantarum* spp. tested by PCR (Kwon *et al*., 2004).

Day[a]	Experimental group			
	LAC	***L. plantarum* B2028**	***L. plantarum* B2028 + LAC**	**Control**
-7	0/6	0/6	0/6	0/6
-1	1/6	4/6	3/6	1/6
4	2/6	3/6	4/6	0/6
10	1/6	4/6	4/6	1/6

[a] Tested throughout the entire feeding trial, during pre challenge week (twice) and post STm SL1344nal^r challenge (twice).

6.2.5.2 *E. coli* spp. changes

As observed for lactobacilli, the inclusion of LAC, *L. plantarum* B2028 or *L. plantarum* B2028 + LAC in the diet had no significant effect on *E. coli* numbers during the first week. On day 4 after challenge *E. coli* numbers in the *L. plantarum* B2028 + LAC treated group were significantly decreased when compared to values in LAC and un-treated control group ($P<0.05$). With the exception of this one time point, no significant differences were observed for *E. coli* numbers at any other time after STm SL1344nal^r challenge. However, there appeared to be lower number of *E. coli* after STm SL1344nal^r challenge across the treatment groups with the exception of the LAC treatment where *E. coli* numbers remained consistently higher than the other groups. These differences were not significant.

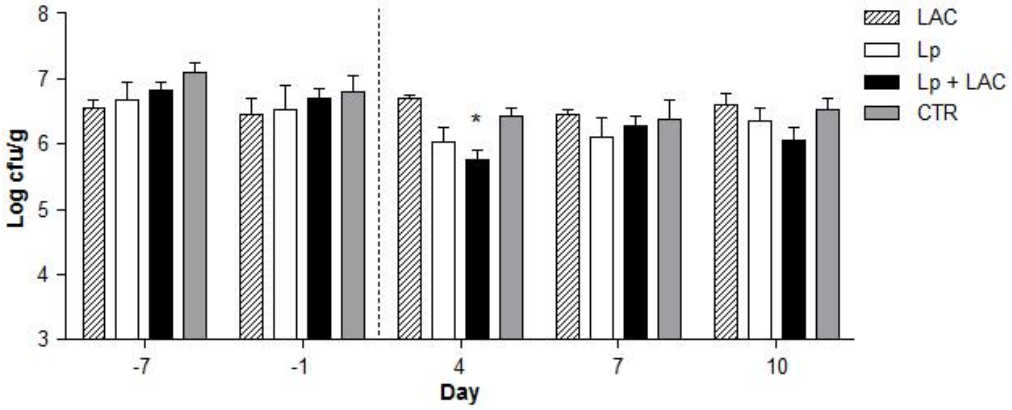


Fig 6.6 Counts of *E. coli* (Log cfu/g) recovered from the faeces of piglets treated with LAC (hatched bar), *L. plantarum* B2028 (white bar), *L. plantarum* B2028 + LAC (black bar) and un-treated control group (grey bar) before and after STm SL1344nal^{r} challenge. Dotted line indicates STm SL1344nal^{r} challenge and day 0 of post-infection period. Presented values are means of six animals in each of the experimental group and the SEM is shown. Values significantly different from the control at the specific time point are indicated by * $P<0.05$.

The ratio of lactobacilli to coliforms has been used previously as an indicator of animal gut health and growth performance (Muralidhara *et al*., 1977; Pollmann *et al*., 1980). It is considered that the higher the ratio, the better the health of the animal gut. The lactobacilli to *E. coli* ratio (L:E) was determined (Figure 6.7) to assess whether treatment with either LAC, *L. plantarum* B2028 or *L. plantarum* B2028 + LAC helped to maintain a favourable L:E relationship. In general, treatments with *L. plantarum* B2028 and *L. plantarum* B2028 + LAC resulted in higher L:E ratio in comparison to un-treated control group and on day 4 after STm SL1344nal^{r} challenge the difference in L:E for the Lp + LAC compared with all other groups was significantly greater ($p<0.05$). Furthermore, at 10 days after challenge the highers L:E ratio was observed in animals treated with *L. plantarum* B2028 + LAC.

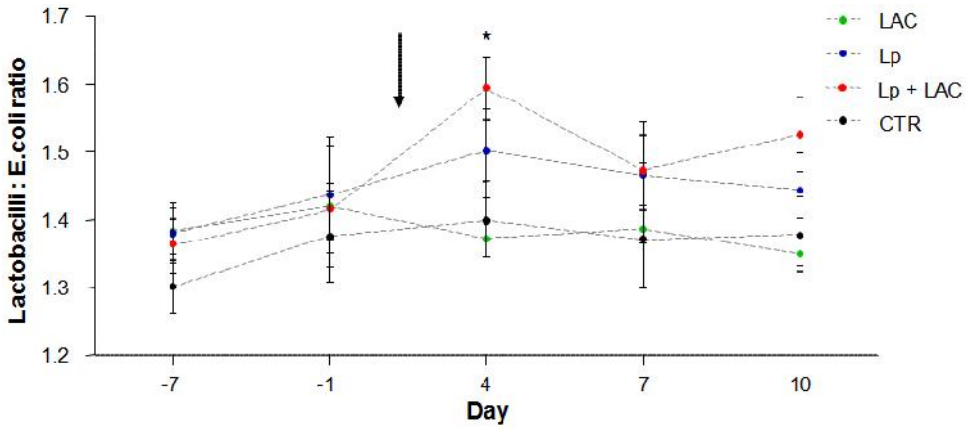


Fig 6.7 Ratio of lactobacilli to *E. coli* recovered from the faeces of piglets treated with LAC (green circles), *L. plantarum* B2028 (blue circles), *L. plantarum* B2028 + LAC (red circles), un-treated control group (black circles) and calculated before and after STm SL1344nal^{r} challenge throughout the study. The arrow indicates STm SL1344nal^{r} challenge and day 0 of post-infection period. Presented values are means of six animals in each of the experimental group and the SEM is shown. Values significantly different from the control at the specific time point are indicated by * $P<0.05$.

6.3 Discussion

Results described in previous chapters demonstrated that *L. plantarum* B2028 or its supernatant reduced the growth, adhesion and invasion into porcine epithelial cells of STm SL1344nal^r. Moreover, Collins *et al*. (2010) reported that this strain modified mucosal barrier function of porcine tissues and promoted host cytoskeletal rearrangements. Probiotics including genera of *Lactobacillus*, *Lactococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus* are live microbial feed supplements and when administered have been reported to confer multiple beneficial effects on pig health, for example improving intestinal microbial balance (Nemcova *et al*., 1999) and reducing the incidence of diarrhoea (Huang *et al*., 2004; Shu and Gill, 2001). One of the ways of enhancing the efficacy of probiotics could be their administration with synergistically acting components (Bomba *et al*., 2002). It was demonstrated in Chapter 3, that LAC supported the growth of *L. plantarum* B2028 and stimulated endogenous lactobacilli within the porcine faecal batch culture system. So the aim of this study was to evaluate the administration effect of LAC, *L. plantarum* B2028 alone or in combination with LAC on *S.* Typhimurium shedding and colonisation, but also to determine associated growth performance parameters of piglets and bacterial population changes including L:E ratio of the bacterial faecal population.

The treatment regimes were incorporated into a diet from one week prior to STm SL1344nal^r challenge until the end of the study. The treatments had no effect on piglet daily weight gain, and physiological performance was not disrupted by any treatment pre-challenge. Throughout the entire study the gain of weight was gradual and increased significantly (P<0.001) in all experimental groups after 2 weeks. Despite STm SL1344nal^r infection it was noticed that in piglets treated with *L. plantarum* B2028 + LAC the ADG showed trend to increase during that second week (P=0.088). It was previously demonstrated by Pollmann *et al.* (1980) that improvement in ADG following inclusion of *L. acidophilus* in ‘starter’ pig diet was dependent upon lactose administration at the same time.

Challenge with 5 x 10^8 cfu/pig of STm SL1344nal^r resulted in the onset of mild clinical infection in all four experimental groups. During the entire post-infection period the body temperature equal to or above 40°C was recorded more frequently in piglets in the un-treated control and LAC group than piglets in *L. plantarum* B2028 and *L. plantarum* B2028 + LAC treated groups, without significance. Animals in all

experimental groups developed mild diarrhea, especially during the first four days, however this parameter did not seem to be significantly affected by any of the treatments. With that in mind, reports in the literature differ in findings and between experimental approaches. For example, previous studies by Szabo *et al*. (2009) demonstrated that administration of *E. faecium* NCIMB 10415 to the diet of weaned pigs and challenged with *S.* Typhimurium increased the incidence of diarrhoea and elevated body temperature in comparison to un-treated group, whereas the contrary was reported by Casey *et al*. (2007).

Colonisation of the piglets by STm SL1344nal^{r}, as assessed by selective culture of faeces, indicated that the majority of piglets in all experimental groups on day 1 after challenge were colonised. Shedding thereafter was intermittent and sporadic and on average only one treatment group, that with *L. plantrarum* B2028 + LAC supplementation, showed statistically significant differences from the control group comparing over entire post infection period. The difficulty with such animal experiments is establishing the correct inoculum to induce sufficient colonisation and clinical symptoms to evaluate any mitigating effects of the various treatments. The strain and inoculum size have been used in these laboratories before successfully in antibiotic treatment studies (Delsol *et al*., 2003). However, the challenge dose may have been too high and thereby overcome any ameliorating effects of the treatments. This would be the case if it assumed that any protective effects of the treatments are modest. Perhaps low dose exposure by in-contact transmission studies may have been a more appropriate challenge model. The limitation on the number of animals for use in this study precluded any investigation of alternative models. In retrospect, it may have been sensible to have studied larger numbers of animals in just two study groups, namely un-treated and *L. plantarum* B2028 + LAC which from the *in vitro* studies seemed most promising. This would have limited teasing out the contributions of LAC but especially *L. plantarum* B2028 alone. Also, whilst every effort was made to ensure the source of the piglets was from a non-vaccinated *Salmonella* free herd, it is possible that some maternal or even induced immunity was in the study group. In retrospect, an assessment of the immune status prior to challenge and during the infection model, even if only by ELISA for humoral circulating antibody, may have enlightening.

Because of restrictions on facility and time, at the end of the study only three pigs from each treatment group were examined *post mortem*. Those which were

shedding *Salmonella* throughout the study were selected for the analysis. Liver, spleen, jejunum, colon, caecum and mesenteric lymph nodes were collected to evaluate STm SL1344nal^r colonisation level. In retrospect, the selection criteria may have introduced bias and perhaps all six animals from the control and from the Lp + LAC treated group may have been more appropriate. The sites of colonisation were very much as expected although evidence for deep tissue colonisation was not gained. The observed colonisation of the digestive track samples and dissemination to mesenteric lymph node samples of STm SL1344nal^r corresponds with the classic and reported sites affected by *S.* Typhimurium (Fedorka-Cray *et al*., 1995; Vieira-Pinto *et al*., 2006). The differences in gut colonisation between treatment groups were again modest. In general, the colonisation differences observed were site dependent; interestingly the lower colonisation level for *L. plantarum* + LAC was seen in the colon and the highest in mesenteric lymph node samples, without significance. Moreover, LAC treatment seemed to be associated with higher colonisation level in those positive animals. These appear somewhat contradictory results but as it was observed in terms of L:E ratios perhaps LAC is metabolised to stimulate the inhibitory effects of Lp and other resident bifidobacteia and lactobacilli.

Treatment with *L. plantarum* B2028 + LAC reduced *Salmonella* shedding (P<0.05) in weaned piglets, which was further linked with increased numbers of *Lactobacillus* spp. As for LAC, increased numbers of lactobacilli were observed, without significance. Administration of both LAC and *L. plantarum* B2028 together modulated the L:E ratio and reduced the numbers of *E coli*. It could be postulated that the *L. plantarum* + LAC did not affect the numbers of *E. coli* whereas the presence of STm SL1344nal^r did, perhaps by some competitive mechanism. However, this seems unlikely given that all the data indicate this treatment suppressed STm SL1344nal^r and therefore reduced numbers of STm SL1344nal^r, are less likely to impact on *E. coli*. It seems more likely that as *E. coli* and STm SL1344nal^r share similar physiologies that the symbiotic or LAC alone may influence colonisation of both as suggested by Guenther *et al*. (2010).

Interestingly, faecal samples subjected to PCR showed the same prevalence of *L. plantarum* positive piglets in groups treated with *L. plantarum* B2028 and *L. plantarum* B2028 + LAC, which is not surprising given the added load of the organisms in the diet. It should however be borne in mind that the PCR is not specific for strain B2028. Clearly, an addition of strain B2028 resulted in enhanced

numbers of *L. plantarum* spp. These data are supported by the findings of Pollmann *et al*. (1980) who observed that in pigs receiving probiotic supplement together with lactose counts of lactobacilli were increased whereas no such effect was observed with administration of the probiotic supplement alone. Letellier *et al*. (2000) also reported increases in Gram-positive rod and coccoid bacteria after probiotic, FOS, and their symbiotic combination during *S.* Typhimurium experimental infection. Their studies showed no changes of pathogen shedding in the faeces, however. *L. plantarum* was present in the pigs prior to the challenge as these organisms were detected in the CTR and LAC groups but in much lower abundance than Lp treated groups. It is possible that LAC stimulated *L. plantarum* growth as the LAC group developed more PCR positive samples than the CTR group.

The reports of *in vivo* efficacy of probiotics and prebiotics in the control of *S.* Typhimurium in pigs are contradictory; this could be strain, dose dependant and diet composition dependant; for example supplementation of single probiotics showed no beneficial effect or greater pathogen excretion and enhanced organ colonisation in pigs challenged with *S.* Typhimurium (Kreuzer *et al*., 2012; Szabo *et al*., 2009). On the other hand using a combination of five probiotic strains observed reductions in *Salmonella* shedding and alleviation of clinical disease outcome (Casey *et al*., 2007).

A trend to reduce the frequency of *Salmonella* excretion in this study was observed, which possibly could be attributable to enhancement of lactobacilli numbers via synergistic effects of *L. plantarum* B2028 and LAC. This could indicate that administration of *L. plantarum* B2028 and LAC together, might prove to be effective in the field as an alternative control measure to control zoonotic *Salmonella* infections in pigs due to gut microbiota modulation and improvement of general animal health especially after weaning. Daily handling of animals for sampling in the studies reported here is an additional stress factor for the piglets which could have enhanced colonisation. Furthermore, the permanent contact of individual animals that were negative and positive for STm SL1344nal^{r} piglets within any one study group and their contact with the contaminated environment could have increased recrudescence of *Salmonella* during this study (Callaway *et al*., 2006; Verbrugghe *et al*., 2011). This would contribute to reducing any significance in tissue colonisation observed among the animals examined. Nevertheless, from the data obtained here we can conclude that LAC is essential to potentiate the probiotic effect of *L. plantarum* B2028 during outcome of *S.* Typhimurium experimental infection and that

longitudinal studies of this synbiotic should be carried out to evaluate further its full potential. Certainly, there is a need to study the immunomodulatory effects of *L. plantarum* B2028 and LAC as these are as yet unknown.

Chapter 7

General discussion

Human infections due to *Salmonella* spp. continuously represent major public health and economic burden (Adams and Moss, 2008; EFSA, 2012; Voetsch *et al*., 2004). Moreover as discussed beforehand (Chapter 1), pork and pork products are important reservoirs of *S.* Typhimurium. Thus, the control of *S.* Typhimurium colonisation in pigs is essential in order to reduce the occurrence of salmonellosis in humans, primarily those attributed to serovar Typhimurium. One of the formerly implemented on-farm approaches to control gastrointestinal pathogens and improve zootechnical performance of animals was the use of AGPs (Dibner and Richards, 2005). It has been postulated that the mechanisms by which AGPs improved the growth promotion was associated with their influence on the balance of GIT microflora, inhibition of pathogens (Dibner and Richards, 2005) and consequences of those changes on immune stimulation (Costa *et al*., 2011; Niewold, 2007). Following the ban on in-feed antimicrobials in the EU (Castanon, 2007) there has been anecdotal evidence for increases in endemic disease and statutory pathogens such as *S.* Typhimurium remain a recognised human health risk factor in the food production chain. Thus improvement of the host's resistance to pathogens by alternative on-farm control strategies has become essential. Hence, the development of pre-, pro- and syn-biotics are clearly understandable approaches, as these represent opportunities to modulate the gut microflora, an already recognised complex and dynamic ecosystem (Xu and Gordon, 2003), to contribute to animal health and performance (Konstantinov *et al*., 2006a). *Lactobacillus* spp. are regarded as one of the major groups of bacteria present in the pig gut (Castillo *et al*., 2006; Konstantinov *et al*., 2004) and are of particular interest due to the growing evidence of the protective and health benefits that they confer in pigs (Bomba *et al*., 1998; Bomba *et al*., 2002; Castillo *et al*., 2011; Konstantinov *et al*., 2006a; Servin, 2004). However, much of this work is empirical

and there remains a need to delve deeper into the mechanisms of protection and health promotion.

Roberfroid (1998) suggested that synbiotics may exert greater beneficial effect to that of individual pre- or probiotics. He argued that prebiotics will stimulate the growth of those organisms capable of metabolising these complex oligosaccharides. Subsequently, fortifying the flora with additional bacterial species that benefit from that metabolism will have greater health and protective impacts than either alone. Thus, one of the aims of these studies was the construction of an effective synbiotic that can successfully be employed to control *Salmonella* colonisation in pigs. Three out of the sixteen probiotic candidates tested in this thesis (Chapter 3), showed strong antimicrobial effect on *S.* Typhimurium growth. However, ultimately, the porcine isolate *L. plantarum* B2028 (Collins *et al*., 2010) was chosen for further studies to comply with the range of selection criteria for new probiotics (Klaenhammer and Kullen, 1999). In addition, a porcine source could imply better survival and ability to colonise the pig GIT. The ubiquitous *L. plantarum* spp. is found in many ecological niches (Lee *et al*., 2011; Plumed-Ferrer *et al*., 2004; Siezen *et al*., 2010) as well as the gastrointestinal tract of human (Ahrne *et al*., 1998; Molin *et al*., 1993) and animals (Collins *et al*., 2010; De Angelis *et al*., 2006; Rodriguez-Palacios *et al*., 2009). Previous studies demonstrated production of bacteriocin by *L. plantarum* spp. (Todorov *et al*., 2010) as well as antimicrobial activity against *Salmonella* (Fayol-Messaoudi *et al*., 2007; Kaushik *et al*., 2009; Makras *et al*., 2006) suggesting that *L. plantarum* isolates were suitable potential probiotic strains for the specific task of impacting on *S.* Typhimurium. Interestingly, because the assay for these studies was control of *Salmonella*, and that alone, it is highly likely that these studies were not fully exploited to assay other potential benefits of the various pro- and pre-biotic combinations assessed.

Having selected *L. plantarum* for use in an *in vivo* model it was necessary to fulfill further selection criteria (Klaenhammer and Kullen, 1999; Saarela *et al*., 2000) such as the ability to resist low pH and bile: these studies were described in Chapter 4. Despite showing sensitivity to the lowest tested pH ranges in the *in vitro* environment, it could still be argued that the presence of feed in the stomach might to some degree confer protection of the probiotic (Conway *et al*., 1987; Desmond *et al*., 2002). Thus, enabling appropriate numbers of viable cells to reach the lower gut and exert their

beneficial properties. Corcoran *et al*. (2005) demonstrated enhanced survival of lactobacilli strains in the presence of metabolisable carbohydrates and hence it could be hypothesized that lactulose might contribute to *L. plantarum* better survival. The ability of lactobacilli to form a biofilm structures on the epithelial surfaces of the oesophagus and the pars oesophagea of the pig stomach (Tannock, 1992), could also be advantageous for the survival in the harsh acidic environment. It remains unclear what numbers need to pass through to the lower gut to exert beneficial effects and the numbers required will depend on their mode action against *S*. Typhimurium. Again, until we have a greater understanding of the mechanisms of action, formulating delivery regimes will be potentially inaccurate estimates. Irrespective of that, protection from acid and bile will enhance the survival of the strain and so approaches such as microencapsulation could be employed (Rokka and Rantamäki, 2010) and which have been demonstrated to improve gastrointestinal resistance of probiotic strains (Del Piano *et al*., 2010). Due to growing awareness of antibiotic resistance, and to conform to the EU legislative framework (discussed in Chapter 1) the *L. plantarum* B2028 resistance profile was assessed showing that *L. plantarum* B2028 is susceptible to seven out of eight tested antibiotics. Nevertheless, prior to any commercial application, the assessment of resistance to quinupristin/dalfopristin and further screening for the presence of resistance genes is required. This was an area of research that for time reasons was not pursued. It is known that prior to commercialisation many probiotic strains are treated to remove resistance genes, especially those that are plasmid mediated that can be lost by simple chemical treatments.

As part of synbiotic to be used in these studies, the prebiotic LAC was selected (Chapter 3). Enhancement of the beneficial gut microbiota with prebiotics and in turn contribution to improved health status and resistance to pathogens is known (Gaggia *et al*., 2010). The ability of LAC to selectively promote intestinal microbiota including bifidobacteria and lactobacilli has been reported extensively in human studies (Ballongue *et al*., 1997; Kontula *et al*., 2002; Tuohy *et al*., 2002). In this study, LAC promoted growth of *L. plantarum* in the pure culture and in fermentations with the complex porcine microflora in which significant increases of *Lactobacillus-Enterococcus* bacteria was observed, which corroborate with the findings of Martin-Pelaez *et al*. (2008). Additionally, *S*. Typhimurium failed to utilise LAC as a sole carbon source (Chapter 3) indicating the combination of *L. plantarum* and LAC would

be the most effective combination in impacting on *S.* Typhimurium. It would be of interest to know whether the breakdown products of LAC from the porcine microflora fermentations would support the growth of *S.* Typhimurium. Simple monosaccharides are likely to be produced but these are likely to be lactose a substrate that salmonellas cannot use, making the selection of LAC logical.

Before the *L. plantarum* B2028 and LAC was evaluated as an intervention against *S.* Typhimurium colonisation in the pig model, it was essential to demonstrate its efficacy using range of the *in vitro* models and to determine the possible mode of action. To test the synbiotic concept for the studies where cell free supernatant was used, LAC was also included in the growth medium as a main carbon source. Numerous studies have reported antagonistic activities of lactobacilli against *Salmonella* due to production of antimicrobial substances, both lactic acid and other compounds (Bernet-Camard *et al*., 1997; Coconnier-Polter *et al*., 2005; Coconnier *et al*., 1997; Coconnier *et al*., 2000; Makras *et al*., 2006; Tejero-Sarinena *et al*., 2012). It was demonstrated (Chapter 3), that the growth inhibition of *S.* Typhimurium by lactobacilli strains was directly correlated to the amount of lactic acid in cell free supernatants and, as mentioned above, the porcine derived *L. plantarum* isolate was one of three lactobacilli that showed strong inhibition of *Salmonella* growth. This inhibitory effect of *L. plantarum* supernatant was confirmed to be pH dependant and could mostly be assigned to the presence of lactic acid (Chapter 5). Nevertheless, this anti-*Salmonella* activity was not solely lactic acid, and so the presence of non lactic acid compound(s) is probable and need further assessment. It is most likely, especially in an *in vivo* environment that various factors, including production of organic acids, non-lactic acid molecules, competition for receptor sites or inhibition by co-aggregation all synergistically contribute towards inhibition of *S.* Typhimurium. It was demonstrated (Chapter 5), that a short pre-exposure of *Salmonella* to *L. plantarum* supernatants reduced the pathogen's ability to invade IPEC-J2 cells but without compromising viability of the *Salmonella*. In fact, it was previously reported that exposure of *S.* Typhimurium to the CFS of *L. acidophilus* LB resulted in temporary impairment of motility of the pathogen, hence it reduced the invasion to human enterocyte-like cells (Lievin-Le Moal *et al*., 2011). Certainly, flagella have been shown to play an important role in the adhesion and invasion of *Salmonella* (Allen-Vercoe and Woodward, 1999; La Ragione *et al*., 2003). It would have been of considerable

interest to explore any impact of *L. plantarum* and/or its CFS upon the motility of *Salmonella*. Low pH will disrupt proton motive force and deplete energy from the motor of the flagella. Also, low pH may cause disaggregation of the protein monomers of flagella changing amplitude or even organelle integrity, thus compromising motility.

Simultaneous co-incubation of *S.* Typhimurium with *L. plantarum* and its cell free supernatant dramatically reduced its ability to invade monolayers and 3D IPEC-J2 cells (Chapter 5). The presence of probiotic supernatant was essential in generating this effect but, interestingly, *L. plantarum* cells together with the supernatant had an even greater inhibitory effect on *Salmonella* invasion. This perhaps suggests a synergistic effect whereby, in addition to inhibitory compounds present in supernatant that effects the invasive phenotype of *Salmonella,* the *L. plantarum* bacterial cells may themselves compete or interfere with the pathogen during the initial attachment. Mappley *et al.* (2011) demonstrated a physical interaction between a lactic acid probiotic and *Brachyspira pilosicoli* that interfered and reduced attachment of the pathogen to host cells. Perhaps physical association between organisms is an important generic control mechanism. This warrants further investigation. However, in this study LAC or washed probiotic cells alone failed to reduce the adherence or invasion *S.* Typhimurium. This suggests that the cell free supernatant plays a primary crucial role in the inhibition and that bacterial cells of *L. plantarum* enhanced the inhibition but only in the appropriate environment, possibly pH or presence of certain metabolites. The importance of the CFS of *L. acidophilus* LA1 for its adhesion was previously demonstrated (Bernet *et al*., 1994). The role of a *L. acidophilus* derived proteinaceous compound present in the supernatant for its adherence was highlighted by the authors. However, the ability of *L. plantarum* to adhere to epithelial cells was demonstrated (Chapter 4), it was not in this instance determined if the adherence is affected by the presence of its own CFS. Thus the exact mechanism of adherence such as mediation by the presence of proteinaceous molecules, mannose adhesins (Adlerberth *et al*., 1996; Bernet *et al*., 1994; Coconnier *et al*., 1992; Greene and Klaenhammer, 1994) or other adhesive structures (Granato *et al*., 1999; Sherman and Savage, 1986) is unclear and would be worth further investigation. However, no protective effect of *L. plantarum* and CFSL against the association of *S.* Typhimurium using porcine IVOC was observed (Chapter 5). Due to the complex nature of this model the effect of the pre and probiotic candidate on the *Salmonella* invasion was not studied more fully, even

though it may be argued that IVOC is a more realistic model system to use. The IPEC-J2 cells that were used for the adhesion and invasion studies were only shown to have the glycocalyx layer on the surface (Schierack *et al*., 2006) as opposed to the porcine jejunal or colonic mucosal tissues used in the IVOC model, which had a mature mucus layer. The key components of the mucus are secreted mucins, but also defensins, lysozyme and secretory IgA (Clamp and Creeth, 1984; Hollingsworth and Swanson, 2004). In studies using human gut mucosal tissues (Haque *et al*., 2004) *Salmonella* was observed in the large numbers in the mucus, but the interaction of the pathogen with the epithelial cells was greatly delayed, suggesting protective role of the mucus layer. Collins *et al*. (2010) in his study, showed however that *L. plantarum* B2028 did not reduce association or cellular pathology induced in the porcine mucosal tissues by *S.* Typhimurium, which was in agreement with the results presented in this study. Nevertheless, the ability to modulate jejunal and colonic mucin levels was demonstrated for this strain. Using human intestinal glycoproteins, Tuomola *et al*. (1999) demonstrated that the ability of lactobacilli to protect against the adherence of *Salmonella* was strain dependent. More in depth studies are therefore needed to evaluate *L. plantarum* interaction with the porcine mucus. It would also be interesting to evaluate production of exopolysaccharides (EPS), that was shown in *L. plantarum* spp. (Tallon *et al*., 2003) and, interestingly, as it was concluded elsewhere that present in the gut EPS could decrease probiotic and increase *Salmonella* adhesion to mucus (Ruas-Madiedo *et al*., 2006).

Inclusion of the of LAC and synbiotics containing LAC in the porcine batch culture system, in part representing the complex conditions of porcine colonic environment, resulted in significant increases of the *Lactobacillus-Enteroccocus* bacterial group. This correlated with the increases in SCFA (Chapter 3). It was then hypothesized that administration of the *L. plantarum* B2028 combined with the ability of LAC to enhance endogenous lactobacilli will impact on pathogen survival. Indeed, further work (Chapter 5) showed that the *L. plantarum* B2028 and LAC synbiotic resulted in decrease in *Salmonella* cell numbers over time. The fermentation of prebiotic was additionally correlated with the gradual increase in SCFA concentrations. *In vivo*, the majority of the SCFA are swiftly absorbed in the colon to be metabolized at various body sites (Cook and Sellin, 1998). Butyrate especially is a key energy source for colonocytes, also important for apoptosis and cell proliferation

(Topping and Clifton, 2001). Interestingly, fermentation of LAC resulted in higher butyric concentration than the other prebiotic tested (Chapter 3) and Martin-Pelaez *et al*. (2008) One limitation of this study was the assessment of bacterial populations by FISH, restricting the information regarding which other members of the pig gut microflora might have been stimulated by administration of the *L. plantarum* and LAC. The synbiotic containing *L. plantarum* and LAC inhibited *S.* Typhimurium survival in the batch culture system but there is scant information on the role of other organisms or other metabolites. Metagenomic approaches may have shed further light on changes in the bacterial population on fermentation of the synbiotic in the fermentation system. Detailed chemical analysis, a metabonomic approach, would have given clues as to shifts in metabolites. These in-depth approaches may have yielded new lines of investigation regarding inhibition of *Salmonella*. Further studies investigating the *Salmonella* membrane damage following time course exposure to *L. plantarum* B2028 supernatant or supernatant collected from the batch culture system might also be helpful. *S.* Typhimurium membrane permeabilization was induced by the CFS of *L. acidophilus* (Coconnier-Polter *et al*., 2005).

The *in vitro* studies/models provided a useful platform to study the anti-*Salmonella* efficacy and the mode of action of probiotics and synbiotics, minimizing animal use. Nevertheless, the *in vitro* systems use do not account for multiple physiological parameters that are going to modulate that effect *in vivo*. It was always the aim of these studies, if financially achievable, to evaluate the prospective probiotic *L. plantarum* B2028 alone or as a synbiotic in a pig infection model. The results generated from the pig study (Chapter 6) corroborated *in vitro* data presented in this thesis and confirmed the superior effect of the probiotic candidate in combination with lactulose in comparison to probiotic or prebiotic alone. Precisely, the higher lactobacilli counts were observed in the group feed the synbiotic and this correlated with the reduced frequency of *Salmonella* shedding. Incidentally, or perhaps more likely as a direct consequence of the symbiotic in the pig diet, the numbers of *E. coli* were also reduced. This is probably unsurprising given the similarities in metabolic capabilities between *Salmonella* and *E. coli* (AbuOun *et al*., 2009). Detection of *L. plantarum* by PCR mainly in those groups receiving pro- and synbiotic might indicate that *L. plantarum* B2028 did colonise the porcine gut, with LAC acting synergistically, possibly enhancing the growth and colonisation of the administered probiotic and possibly

similar strains that were already resident in the gut flora. It is to be presumed the beneficial effects which, in this study was primarily the reduction of intestinal carriage of *S.* Typhimurium, were mediated by lactobacilli. However, as discussed above, without a full analysis of the entire gut population and its metabonome it is not possible to say this authoritatively. However, the addition of fermentable carbohydrates supported growth specific lactobacilli in the gut of weaning piglets (Konstantinov *et al*., 2004) and, similar to the findings of the study reported here. The administration of *L. paracasei* in combination with maltodextrins, FOS or polyunsaturated fatty acids enhanced pathogen reduction in the pig (Bomba *et al*., 2002).

The porcine intestinal microbial community represents a very complex and balanced ecosystem (Leser *et al*., 2002) that under stress conditions, such as during weaning or infection, might get considerably disturbed (Pieper *et al*., 2008). Feed additives that mitigate against such gut disturbances may also protect against the worst effects of change or infections. Indeed, it has been reported that probiotics are most effective in the young pigs, particularly when the gut microlora is disturbed at weaning (Stavric and Kornegay, 1995). Even a single oral administration at the time of weaning was shown to modify the intestinal microbiota that benefitted the health status of the pig (Pieper *et al*., 2009).

The protective effects of pro- and prebiotics may be exerted against pathogens in many ways; competition for nutrients and receptors, production of antimicrobial compounds, including organic acids that create unfavorable niche (Servin, 2004). In the future it would be interesting to evaluate the effect of those treatments and correlation on complex bacterial populations, the SCFA concentrations in the gut contents and metabolites changes in the gut contents, body fluids and various tissues. Perhaps to further enhance the effect of synbiotic combinations used in this study, future studies should concentrate on isolation of other potential porcine probiotic strains that could be used in conjunction with these synbiotics. The findings of Casey *et al*. (2007) could indicate that multiple species are required to confer best protective effect against *Salmonella* infection. Recent studies reported isolation of *L. sobrius* a common commensal present in the gastrointestinal tract of piglets (Konstantinov *et al*., 2006b) that was shown to exert protective effect against *E. coli in vitro* (Roselli *et al*., 2007) and *in vivo* (Konstantinov *et al*., 2008). It may be attractive to include additional

prebiotics, such as MOS that acts as a receptor analog, so the pathogen inhibition and host health improvement at the gut level can be mediated via various mechanisms. Previous studies seems to support the idea of supplementation of probiotics to pregnant sow and neonatal piglets (Genovese *et al.*, 2000; Taras *et al*., 2005) and perhaps future studies should evaluate inclusion of *L. plantarum* and LAC to sows and their litters.

Collectively, the *in vitro* and *in vivo* studies presented in this thesis, indicated that the synbiotic that comprised a porcine isolate of *L. plantarum* and lactulose was more effective to modulate the selected bacterial populations in comparison to *L. plantarum* or lactulose alone. Furthermore, an *in vitro* anti-*Salmonella* activity and the ability to reduce the frequency of *S.* Typhimurium shedding in the trial study was demonstrated. The evidence gained suggests that this synbiotic approach could be applied to improve gut health and as a control strategy for *S.* Typhimurium in pigs. Nevertheless, longitudinal studies in the field are required that should also focus on the effect of *L. plantarum* and lactulose administration on the immune response in pigs treated with this synbiotic.

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