Bifidobacterial Physiology and Metabolism in the Gut Environment.

Ollscoil na hÉireann, Corcaigh

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DECLARATION

I hereby declare that the research presented in this thesis is my own work and effort, and that it has not been submitted for any other degree, either at University College Cork or elsewhere. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

This work was completed under the guidance of Prof. Douwe van Sinderen at the School of Microbiology and APC Microbiome Ireland, Food Science Building, University College Cork.

Signature:

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Chapter II**: Kelly, SM., O'Callaghan J., Kinsella, M. & van Sinderen D.** (2018). Characterisation of a Hydroxycinnamic Acid Esterase from the *Bifidobacterium longum* subsp. *longum* taxon. Front Microbiol, 10.3389/fmicb.2018.02690.

Chapter V: **Kelly, SM., Lanigan N., O'Neil, I., Bottacini F., Lugli, GA., Viappiani, A., Turroni, F., Ventura, M. & van Sinderen, D.** (2020). Bifidobacterial biofilm formation is a multifactorial adaptive phenomenon in response to bile exposure**.** Sci Rep, https://doi.org/10.1038/s41598-020-68179-9.

ABBREVIATIONS

ABC – type transporters: ATP-Binding Cassette Transporters

AH: Alimentary Health

AI: Auto inducer

APC: Alimentary Pharmabiotic Centre

ArbPCR: Arbitary Primed PCR

ATCC: American Type Culture Collection.

AOS: Arabino-oligosaccharides

AX: Arabinoxylan

AXOS: Arabinoxylo-oligosaccharides

Blast: Basic Local Alignment Tool

BlastN: Nucleotide Based Basic Local Alignment Tool

BlastP: Protein Based Basic Local Alignment Tool

CAZy: Carbohydrate Active Enzyme.

CCUG: Culture Collection University of Gothenburg

CDC: Chenodeoxycholic acid

CFU: Colony Forming Units.

Cm: Chloramphenicol

DNA: Deoxyribonucleic acid

DNase: Deoxyribonuclease

DP: Degree of Polymerisation.

eDNA: Extracellular Deoxyribonucleic acid

EM: Extracellular Matrix

EMSA: Electrophoretic Mobility Shift Assay

EPS: Extracellular Polysaccharide

fRCM: Filtered Reinforced Clostridial Media

F6PK: Fructose 6 phosphate Phosphoketolase

GC: Glycocholic acid

GDC: Glycodeoxycholic acid

GH: Glycosyl Hydrolase

GIT: Gastrointestinal Tract

GM17: Glucose – M17 media

GPH: Glycoside Pentoside Cation Symporter Family Transporter

HCA: Hydroxycinnamic acid

HG: Homogalacturonan

HMOs: Human Milk Oligosaccharides.

HPAEC–PAD: High Performance Anion Exchange Chromatography – Pulse Amperometric Detection

HPLC: High Performance Liquid Chromatography

iPCR: Inverse PCR

IPTG: Isopropyl β-D-1-thiogalactopyranoside

ISAPP: International Scientific Association for Probiotics and Prebiotics

JCM: Japan Collection of Microorganisms

kDa: Kilodaltons

LacI: Lactose inhibitor type DNA transcription factor

LAB: Lactic Acid Bacteria

MEME: Multiple Em for Motif Elicitation, Motif based Sequence Analysis Tool

MFS: Major Facility Superfamily Membrane Transporter

MOPS: Morpholinepropanesulfonic Acid

MRS: de Mann, Rogosa and Sharpe Medium

mMRS: Modified de Mann, Rogosa and Sharpe Medium

MW: Molecular Weight

NCC: Nestle Culture Collection.

NCIMB: National Collection of Industrial and Marine Bacteria

O.D.: Optical Density

PEP-PTS: Phosphoenolpyruvate – Phosphotransferase System

Pfam: Protein family database

***p*-Np:** *para* Nitrophenol

PWM: Position Weight Matrix

RCA: Reinforced Clostridial Agar

RCM: Reinforced Clostridial Media

RGI: Rhamnogalacturonan I

RGII: Rhamnogalacturonan II

RNA: Ribonucleic Acid

RNAseq: RNA sequencing

SCFA: Small Chain Fatty Acids

SDS – PAGE: Sodium dodecyl Sulphate Poly Acrylamide Gel Electrophoresis

Signal P: Protein Secretion Signal Prediction Software

TC: Taurocholic acid

TDC: Taurodeoxycholic acid

Tet: Tetracycline

TF: Transcription Factor

Tris – HCL: Tris (hydroxymethyl)aminomethane - hydrochloride

UCC: University College Cork

v/v: volume/volume

XOS: Xylo-oligosaccharide

w/v: weight/volume

ABSTRACT

Bifidobacteria represent Gram-positive gut commensals of mammals, birds and insects. Certain bifidobacterial species are associated with various health benefits if present in adequate amounts in the human gastro-intestinal tract (GIT). Bifidobacteria are highly prevalent and abundant in the infant gut, though they decrease in abundance with increasing age of their human host. Bifidobacteria must overcome many challenges in order to survive in the human gut, such as competition by other gut microbes, exposure to bile salts/acids and a fluctuating pH, and nutrient starvation. One key factor to bifidobacterial survival in the gut environment is the ability of members of the genus *Bifidobacterium* to metabolise complex carbohydrates indigestible to the human host. In the infant gut such carbohydrates are supplied in breast milk, whilst in the adult diet complex carbohydrates are often derived from plant-based oligosaccharides ingested from the diet. In particular, members of the *Bifidobacterium longum* subsp. *longum* taxon are associated with plant-derived poly- and oligo-saccharide utilisation. The research described in this thesis studied the metabolism of certain plant-derived oligosaccharides by different strains in the *B. longum* subsp. *longum* taxon. Bile salts possess major anti-microbial activity and act by disrupting the integrity of the bacterial cell membrane. Concentrations of bile salts/acids are highest in the small intestine and starting from the duodenum a decreasing gradient exists along the GIT with lowest bile concentrations in the large intestine. Therefore, if bifidobacteria are ingested as an active ingredient of a functional food, they encounter and must be able to survive bile stress if they are to be effective as a probiotic. This thesis examined biofilm formation of *Bifidobacterium breve* UCC2003 in response to bile stress and assessed this ability in terms of survival and genetic requirements.

Chapter II of this thesis focused on an *in silico* annotated hydroxycinnamic acid esterase encoded within a genetic locus present in *B. longum* subsp. *longum* NCIMB8809 suspected to be involved in plant-derived poly/oligosaccharide utilisation. Both this locus and encoded esterase were found to be conserved amongst several strains of the *B. longum* subsp. *longum* taxon. Through a number of experiments the function of the esterase was proven and the enzyme was characterised. Therefore, it is likely this esterase cleaves off

hydroxycinnamic acids commonly present as substituents on arabinose moieties present in certain plant-derived poly/oligosaccharides.

Chapter III investigates the enzymatic degradation of plant-derived poly/oligosaccharides by *B. longum* subsp. *longum* NCIMB 8809. This strain was found to grow on various plant-derived glycans including arabinoxylan, arabinogalactan and XOS. In this chapter, three glycosyl hydrolase (GH) 43 enzymes, from a presumed plant-oligosaccharide cluster described in Chapter II, were found to possess exo-α-L-arabinofuranosidase or α-endo-arabinanase activity. Furthermore, these enzymes were found to elicit activity against arabino-oligosaccharide (AOS) substrates. Therefore, a novel locus involved in AOS plant-oligosaccharide utilisation was identified in certain members of the *B. longum* subsp. *longum* taxon.

Chapter IV describes the investigation of a LacI-type regulator, designated here as AauR, present in the *aau* locus of *B. longum* subsp. *longum* CCUG 30698 and predicted to regulate transcription of the *aau* locus encoding various GH43 enzymes. The consensus AauR recognition motif previously predicted for this regulator in another *B. longum* subsp. *longum* representative was indeed also shown to be present in strain CCUG 30698. This study showed that AauR binds to its predicted operator sequences located at specific positions within the *aau* locus. Therefore, AauR is presumed to transcriptionally regulate this locus, likely mediated through an as yet unidentified effector.

Chapter V explores biofilm formation of *B. breve* UCC2003 in response to high concentrations of bile and bile salts. Through experimentation it was found that various factors are involved in biofilm formation including extracellular polysaccharide (EPS) production, LuxS and fatty acid biosynthesis. The formation of biofilm was found to be protective against high concentrations of bile. Extracellular DNA production, proteins and EPS were all found to impact on biofilm formation. Therefore, the formation of biofilms in bifidobacteria is presumed to constitute a multi-factorial process in response to high concentrations of bile.

This thesis represents novel information on the metabolism of plant-derived oligosaccharides, specifically HCA removal, AOS metabolism and AOS transcriptional regulation in *B. longum* subsp. *longum* taxon. The mechanism of biofilm formation in *B.*

breve UCC2003 was also investigated and this is the first report on the molecular players important for biofilm formation in bifidobacteria.

Future research is required to further elaborate on the enzymatic steps that are required for AOS and arabinan metabolism by various *B. longum* subsp. *longum* strains in order to explain strain-specific differences on the basis of encoded GH activities. Also, further detailed analysis of transcriptional regulation of the *aau* genetic locus is needed to identify the molecular effector of the AauR regulator, as well as the precise location of the relevant promoter sequences and the manner by which AauR controls transcription of its target genes. Finally, additional experimentation is required to further investigate the biological roles of bifidobacterial biofilm formation in the gut. It will in particular be important to learn how biofilm formation is triggered by certain bile components and to what extent biofilm formation is important for colonisation and survival under *in vivo* conditions. Ultimately, the generated knowledge on plant glycan metabolism and biofilm formation will contribute to our understanding of how a bacterium can take up residence and survive in a very crowded and sometimes hostile environment.

Chapter I

General Introduction

This chapter has been accepted for publication as a review in Frontiers in Microbiology:

Kelly, SM., Munoz Munoz, J. & van Sinderen D. (2020). Plant Glycan Metabolism by Bifidobacteria. Front Microbiol, accepted for publication.

Dr. Jose Munoz Munoz[1] generated Figure 1.5 of this chapter.

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1.1. Abstract

Most members of the genus *Bifidobacterium* are gut commensals which represent Gram-positive, non-motile, saccharolytic, non-sporulating, anaerobic bacteria. Many bifidobacterial strains are considered probiotic and therefore are thought to bestow health benefits upon the host if present in sufficient viable numbers in the gastrointestinal tract (GIT). Bifidobacteria are highly abundant among the gut microbiota of healthy, full term, breast-fed infants, yet the relative average abundance of bifidobacteria tends to decrease as the human host ages. Because of the inverse correlation between bifidobacterial abundance/prevalence and health, there is an increasing interest in maintaining or restoring bifidobacterial populations in the infant, adult and elderly gut. In order to colonize and persist in the GIT environment, bifidobacteria must be able to metabolise complex dietary or host-derived carbohydrates and be resistant to various environmental challenges of the gut, in particular bile stress. This is not only important for the autochthonous bifidobacterial species colonising the gut, but also for allochthonous bifidobacteria provided as probiotic supplements in functional foods. For example, *Bifidobacterium longum* subsp. *longum* is a taxon associated with the metabolism of plant-derived poly/oligosaccharides in the adult diet, being capable of metabolising hemicellulose and various pectin-associated glycans. Many of these plant glycans are believed to stimulate the metabolism and growth of specific bifidobacterial species and are for this reason classified as prebiotics. There is also a gradient of bile along the GIT and bifidobacteria have adopted various approaches to respond to bile stress, including biofilm formation. In this review, we will discuss the general features of the gut microbiota, and elaborate on how factors, such as age, diet and the physical GIT environment, may influence the gut microbiota composition. Furthermore, the mechanisms by which bifidobacteria colonize and persist in the GIT environment will be covered with an emphasis on carbohydrate metabolism, including mechanisms of poly-/oligosaccharide degradation and uptake, as well as its associated regulation, whilst also focusing on the bifidobacterial bile stress response.

1.2. Introduction

General features of the Gut Microbiota

The human gastrointestinal tract (GIT) contains trillions of microorganisms which represent all three domains of life, while also including non-living biological entities such as viruses and bacteriophages (1). This hugely diverse collection of intestinal microorganisms is termed the gut microbiota (2). Higher eukaryotes such as mammals, birds and insects all contain a particular assembly of microorganisms in their GIT and the presence of this gut microbiota is essential for normal intestinal development and a properly functioning physiology of the host (3). The GIT is the most densely populated body site with an estimated bacterial biomass that is in excess of 1.5 kg, although this biomass has also been estimated to be as low as 0.2 kg (4, 5). Notably, the composition and density of the gut microbiota varies along the GIT **(Fig. 1.1A)**. The oral cavity contains an abundance of microbial species of high diversity (~700 different prokaryotic taxa) (6), whereas, due to the very acidic conditions, the stomach possesses a low bacterial load of 10^2 - 10^3 cfu/ml with a distinct microbial composition (7, 8). Compared to the large intestine the proximal small intestine has a rather low abundance of microbial species, estimated at $<10^3$ cfu/ml (9), due to high immune activity, fast transit time and bile secretion, although the microbial density gradually increases towards the distal ileum, where it reaches density levels that similar to those found in the large intestine (10). The largest biomass of the GIT residues in the lumen of the colon and caecum, where its contents are rich in glycans and where bile concentrations are much lower than in the small intestine. In this review, we will focus on the GIT community composition in the colon. Despite the large quantity of microorganisms present in the large intestine, the majority of the gut-associated bacteria taxonomically belong to just five phyla: Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Verricomicrobiota (11-13). This contrasts with the microbiota of other human body sites, such as the skin, which in comparison is typically more diverse (14). The gut microbiota of a given individual may be classified as belonging to one of three enterotypes: enterotype 1, which is dominated by the *Bacteroides* taxon; enterotype 2, dominated by the *Prevotella* taxon; or enterotype

3, dominated by the *Firmicutes* taxon (15). However, the concept of enterotypes has been controversial and rather than stratification of the gut microbiota into these discrete groups it has been suggested that it is more likely that the gut microbiota is better represented as gradients of these groups (16-18). Members of the gut microbiota can be autochthonous, i.e. indigenously resident, or allochthonous, in which case they are perceived as transient GIT inhabitants, including pathogens. The gut microbiota composition between individuals is highly variable, although the overall species/strain composition within an individual's gut microbiota, once an adult microbiota has been established, does not appear to vary considerably over time (14, 19, 20).

The gut microbiota interacts with and influences the host, and has been claimed to be responsible for or contribute to vitamin production (21), host immune development (22), pathogen exclusion (23), glycan metabolism (24, 25), neurotransmitter signalling (26) and short chain fatty acid (SCFA) production (27). Certain bacterial groups, such as bifidobacteria and lactobacilli, represent components of the gut microbiota that are believed to bestow beneficial effects upon the host and are for this reason termed 'probiotic' (28). The International Scientific Association for Probiotics and Prebiotics (ISAPP) definition of a probiotic is any live microorganism that bestows a health benefit upon its host when ingested in adequate amounts (28). This does not include microorganisms associated with fermented food production, for instance *Streptococcus thermophilus*, which is employed for yogurt production, and which does not have any reported health benefits.

For obvious reasons, it is believed that an increase in abundance of probiotic species in the gut will benefit host health. On the other hand, if a disturbance in the microbiota invokes a compositional change in the community to allow unfavourable species to dominate, it may change the overall community-mediated immune balance to cause inflammatory bowel diseases (IBD), such as ulcerative colitis (UC) or Crohn's disease (29, 30). For example, loss of butyrate-producing species such as *Faecalibacterium prausnitzii* and *Roseburia hominis* is reported in individuals with UC or Crohn's disease (31, 32). Antibiotics can also disturb the microbiota and although it has been reported that the community generally recovers to almost the pre-treatment state, it may still be

permanently altered from the initial composition (33-36). It must also be noted that there is no defined 'healthy microbiota' and it has been thought that the term 'dysbiosis', i.e. a state representing an altered, unhealthy microbiota, has been assigned too readily by supposedly undesirable changes in taxonomic presence or absence of certain microbial groups, although such changes did not appear to alter the overall metabolic abilities encoded by the microbiota (37, 38). Indeed, it has been reported that the combined metabolic functionalities encoded by the gut microbiota are well conserved despite inter-individual variation in microbiota composition (39, 40).

One strategy to remedy a 'disturbed' microbiota and/or reduce symptoms of GIT disorders, like IBD or irritable bowel syndrome (IBS), is to ingest 'probiotic' bacteria, which, when incorporated in so-called functional foods, must be regularly supplemented in sufficient quantities (28, 41, 42). However, stable engraftment of probiotic strains appears to occur in cases where the probiotic species introduces a novel metabolic ability or represents a species not present in the resident microbiota (43). Therefore, there is an interest in modulating the gut microbiota activities in order to treat 'dysbiosis' and (associated) GIT disease states.

Development of the gut microbiota as we age.

It is generally thought that colonisation of the human gut commences at birth. However, several studies have reported that infants are already exposed to microbes in the womb by the placental microbiome (44-46). This topic remains contentious as it has been suggested that these results are a result of sample collection contamination or contamination from DNA extraction kits, the latter often referred to as the 'kitome' (47-51). Recent research reported the presence of bacterial DNA in the amniotic fluid and detection of SCFAs in the meconium (52, 53), although another recent study reported no evidence of a placental microbiome (54).

Full term, vaginally born infants possess a microbiota that resembles that of their mother's vaginal microbiota and is associated with higher counts of bifidobacteria and *Bacteroides* than infants born by caesarean section (55, 56). Infants born by caesarean section on the other hand have been found to harbour a gut microbiota with species that are typically associated with the skin, such as staphylococcal species (55), while lacking

certain taxa, e.g. *Bacteroides*, and being more prone to colonisation by opportunistic pathogens (57). One study has shown that vaginally born infants have a higher abundance of lactobacilli that are presumed to originate from the mother's vaginal microbiome (55), although another study did not report this and instead saw the transmission of *Bacteroides* from mother to infant (57). Transmission of bifidobacteria and/or bifidobacterial species/strains shared by mothers and infants has also been reported (58-60). However, the effects of delivery mode on gut microbiota composition appear to be short term with observed differences disappearing after 6 to 12 months (61). In general, the inter-individual infant microbiota composition tends to fluctuate more when compared to that of adults (62). The (62-64). In contrast, the adult gut microbiota, as discussed in the previous section, is more stable over time when compared to infants; bifidobacteria are present but tend to be lower in abundance (3 % - 6 %) and bifidobacterial abundance appears to decline with age (65, 66).

The elderly gut microbiome (individuals older than 65 years) has been shown to be distinct from that of other (i.e. non-elderly) adults, and to contain a lower abundance of the phylum Actinobacteria, which include bifidobacteria (66-68). The elderly gut microbiota, similar to the adult gut microbiome, elicits a greater inter- than intra-individually diversity, but in contrast was shown to be generally more unstable and possessing an increased relative abundance of *Bacteroidetes* (67, 68). However, greater instability and cumulative changes were observed for elderly individuals who resided in long term care as compared to individuals who were still living in the community (68, 69). Elderly subjects may also possess a gut microbiota with an increased abundance of the pathogen *Clostridium perfringens* (70). Interestingly, the gut microbiome of centenarians differs greatly from that of (other) elderly, with the former possessing an increased abundance of Proteobacteria and differences in the Firmicutes clostridial subgroup clusters, an example being *Eubacterium linosum*, which has been proposed as a biomarker species for longevity (71). Therefore, the gut microbiota develops and changes as the human host ages and understanding how and why the microbiota changes as we age may allow intervention and modulation of the microbiota to prevent and resolve disease states.

Many factors influence the composition of the gut microbiota and as mentioned above microbiota changes occur as we age, while diet, the physical environment of the GIT, the immune system, xenobiotics, host genetics, disease state and various other aspects are known to cause compositional changes of the gut microbiota (72). In the following sections we will focus on how diet and the physical environment of the GIT affect microbiota composition.

The effect of diet on gut microbiota composition.

Diet is a major factor in determining microbiota composition. It has been shown that even short-term dietary interventions may rapidly alter the gut microbiota (73). Dietary fibres are carbohydrates, including lignin, with 10 or more monomeric subunits that cannot be hydrolysed by enzymes found in the small intestine of humans. Dietary fibres include i) naturally occurring carbohydrate polymers in consumed foods, ii) carbohydrates that are extracted physically, chemically or enzymatically from raw foods, such as fresh fruit and vegetables, and that have a physiological benefit, and iii) synthetic carbohydrates with a physiological benefit. Plant carbohydrate polymers under 10 monomeric subunits, but between a degree of polymerisation (DP) of 3 and 9, can be classified as dietary fibres but this is decided at a national level and not by the Codex Alimentarius Commission (74). Glycan is a broader term that refers to complex carbohydrates (polymers and oligosaccharides), which are generally indigestible to the human host, yet can be metabolised by the gut microbiota, and may include carbohydrates with less than 10 monomeric units, which may result from dietary fibre degradation by the gut microbiota (75). Dietary fibres/glycans are found in the plant cell wall **(Figure 1.2)** (75) and are common components in cereals (76, 77), fruit (78, 79), vegetables (80, 81) and red grapes (82), thus being a typical constituent of the human diet; examples of these are fructo-oligosaccharides (FOS), inulin, pectin, arabinoxylan, xylan, arabinan, starch and lignin (83). This review will focus on plant-derived hemicellulose and pectic glycans, the structures of which will be described in more detail below. The microbial degradation of FOS, inulin and (indigestible) starch are not discussed in this review and the reader is therefore referred to a number of specific reviews on this subject (75, 84). Although many plant-derived glycans cannot be digested by the human host itself,

individual members of its gut microbiota encode a plethora of different, 'Carbohydrate Active Enzymes' (CAZyme) which facilitate the degradation of these complex, dietary fibre-derived saccharides (85). CAZymes are represented by (i) glycosyl hydrolases (GHs) which are responsible for the hydrolysis of glycosidic bonds, (ii) glycosyl transferases (GTs), that form glycosidic bonds, (iii) polysaccharide lysases (PLs), responsible for the non-hydrolytic cleavage of glycosidic bonds, (iv) carbohydrate esterases (CEs), that cleave ester bonds, and (v) auxiliary activities (AAs), which represent redox enzymes that act in concert with other CAZymes (86). There are currently 168 GH families, 111GT families, 40 PL families and 18 CEs recognised in the CAZy database (http://www.cazy.org). The CAZyme profile of gut microbes is enriched for plant glycan-degrading enzymes, and despite community variation, this profile is conserved depending on the particular body site, indicating that the microbiome is adapted to carbohydrates present in the local GIT environment (87).

The microbiota composition of individuals from a 'Western' society in Europe and the USA, is distinct from that of rural communities in Africa and South America with a notable decrease in microbiota diversity in the former group (62, 88, 89). One of the main differences between these groups is the consumption of high amounts of plant fibre in rural communities; for instance, rural children from Burkino Faso aged between 1-2 years and 2-6 years consumed 10 g/day (2.26 % of total diet/day) and 14.2 g/day (3.19 % of total diet/day) of fibre, respectively, whereas children from the EU aged between 1-2 years and 2-6 years consumed 5.6 g/day (0.67 % of total diet/day) and 8.4 g/ (0.9 % of total diet/day), respectively (88). Other studies comparing the Hadza hunter-gatherers with 'Western' populations recorded that less than 10 % of the Western diet consists of plant fibre, whilst the Hadza diet is known to predominantly consist of plant-based, high-fibre foods (89, 90). Consumption of fibre alters the gut microbiome in becoming enriched for enzymes involved in the degradation of plant-derived, complex poly/oligo-saccharides (62, 88, 91). Diets lacking in such fermentable carbohydrates may cause depletion or even extinction of corresponding fibre-metabolising microbial species in the gut (92). One way to modulate the gut microbiota is by dietary means through the supplementation of so-called prebiotics, which are defined as 'a substrate that is selectively utilised by host microorganisms conferring a health benefit' (93). Among the first prebiotics that were

included in foods as functional ingredients were complex carbohydrates such as FOS and galacto-oligosaccharides (GOS) (94). Prebiotics that specifically stimulate bifidobacterial growth are termed 'bifidogenic' (94, 95). The definition of prebiotic, however, does not include fibres, such as pectin or xylan, which stimulate growth of a broad range of species in the GIT (93). It should also be noted that the ISAPP widened the definition of prebiotics to not only include certain non-digestible oligo/polysaccharides, but also to incorporate other beneficial molecules such as polyphenols (93). Therefore, while some plant fibres are not 'prebiotic' themselves they may still contain prebiotic components such as polyphenols. The related term synbiotic represents a combination of a probiotic organism and a corresponding prebiotic, being supplied together to elicit a synergistic effect through increased abundance of the probiotic and its associated beneficial effect(s) (96, 97).

Short chain fatty acids (SCFAs) are carboxylic acids that possess aliphatic chains between one and six carbons in length (98); butyrate, acetate and propionate represent SCFAs that are predominantly produced through microbial fermentation of carbohydrates, including those constituting dietary fibres/glycans (75, 99, 100). SCFAs have been shown to elicit various health benefits: butyrate is the main source of energy for enterocytes in the colon (101), while propionate and acetate induce apoptosis in colon cancer cell lines (102, 103) and loss of butyrate-producing species has been reported in the microbiome of patients with GIT disorders such as IBD (31, 32). Furthermore, a mouse model has shown that the G protein-coupled protein receptor Gpr41 is activated by propionate, pentanoate, butyrate and acetate, and this interaction influences energy harvest from the diet and host adiposity (104, 105). Moreover, murine diets that had been formulated so as to allow high acetate or butyrate production by microbial fermentation were found to protect against diabetes by decreasing autoreactive T cells and increasing the number of regulatory T cells, respectively, while a diet that produced both high acetate and butyrate levels reduced IL-21, a diabetogenic cytokine (106). Acetate production by *B. longum* subsp. *longum* from fructose fermentation has been shown to cause anti-inflammatory effects and/or to block epithelial apoptosis in a murine model, thereby preventing translocation of the Shiga toxin produced by *Escherichia coli* O157:H7 into the bloodstream, and in this way providing protection against this gut pathogen (23). In addition, lactate, an organic acid (but not a SCFA), is a major metabolite produced during

microbial metabolism of fibres that is important due to its metabolic conversion into butyrate by particular gut commensals (107). SCFAs are also metabolised by certain members of the microbiota and are important in metabolic cross-feeding between species (108, 109). For example, *B. longum* subsp. *longum* is known to metabolise arabinoxylo-oligosaccharides (AXOS) producing acetate, which *Eubacterium rectale* can then metabolise to produce butyrate (109). Therefore, dietary modulation of the microbiota is an interesting route to promote the abundance and metabolic activity of particular probiotic species in the GIT in order to increase production of SCFAs/lactate producing microbial species in the GIT. The recently coined concept of 'postbiotics' is relevant in this context as it refers to any beneficial metabolic end product, such as SCFAs, or microbial component, for example lipids, teichoic acids, peptides or peptidoglycan fragments, released following lysis of a (probiotic) bacterium (110). For instance, the supernatant of a *F. prausnitzii* culture was shown to elicit anti-inflammatory activity by reducing NF-κB and IL-1β in Caco-2 cells and reduced the severity of trinitrobenzenesulphonic acid-induced colitis in mice (111). The cell free supernatants of cultures of *Propionibacterium acidipropionici* or *Propionibacterium freundenreichii*, which contain the SCFAs acetate and propionate, induced cell apoptosis in colorectal carcinoma cell lines (103).

Dietary fibres may also be decorated with hydroxycinnamic acids (HCAs), such as ferulic acid or chlorogenic acid (112). HCAs are phenylpropanoids which are composed of a nine carbon skeleton, include an aromatic ring with several hydroxyl groups, and which require esterases to remove them from a carbohydrate backbone (113-116). HCAs that are in free form are absorbed in the small intestine (117). It should also be noted that HCAs that are linked to plant-derived polysaccharides, are not readily absorbed and therefore reach the colon (118). HCAs can only be released and made bioavailable by gut microbiota members that produce esterases to cleave the ester bond between the HCA and polysaccharide (119). HCAs are viewed as beneficial due to their properties as anti-oxidants (116, 120). The gut microbiota has also been shown to bio-transform HCAs by decarboxylation and reduction (121-124), for example ferulic acid may be decarboxylated to form caffeic acid and/or reduced to form dihydroferulic acid (122). HCAs can act as external electron acceptors for various heterofermentative

bacteria, such as *Weissella cibaria*, *Lactobacillus brevis*, *Lactobacillus curvatus*, and *Lactobacillus rossiae* (125). For these bacteria HCA supplementation to their growth medium was shown to increase acetate kinase activity, thereby causing enhanced acetate production, an increased NAD(+)/ NADH ratio, and higher intracellular ATP levels. HCAs are also bactericidal and HCA-mediated damage to the cell membrane of *Lactobacillus plantarum* has been observed by transmission electron microscopy (126) and of *Dekkera* yeast species (127). HCAs also inhibit growth of certain gut pathogens, such as *C. perfringens* (128), presumably through induced membrane damage. The definition of 'prebiotic' may in principle allow for the inclusion of HCAs although it is still unclear whether the positive effects of HCAs also apply to the human GIT environment and therefore in depth, mechanistic studies are needed to corroborate the purported benefits of these compounds (93, 129).

Diet also has a major impact on the infant microbiome. For instance, breast-fed infants generally have a significantly higher abundance of bifidobacteria and lactobacilli compared to their non-breast-fed counterparts (64, 130). Breast milk itself may contain viable bifidobacteria, while it is rich in so-called human milk oligosaccharides (HMOs) (131, 132), which are highly specific growth substrates for particular bifidobacteria (133, 134). It was also found that the cessation of breast feeding and introduction to solid foods, referred to as weaning, is thought to induce changes to a more adult-like microbiome in infants (64, 130).

The gut environment – a compartmentalised environment.

Another factor that influences the microbial community composition is the gut environment itself. It has previously been shown that the physical environment to which microbes are exposed shapes the microbial community found there (14). It must also be noted that different anatomical sites in the gut harbour particular microbial species (13). For example, the actual number of microbial species present in the small intestine is lower compared to that in the large intestine. It has also been observed that the microbial community associated with the mucosal layers of the GIT is different from that of the gut lumen (135). This is due to various factors such as oxygen content, pH, presence of bile acids, the specific immune response at particular sites and metabolic resources available

(Fig. 1.1A). Due to space considerations, this review will focus on bile acid and carbohydrate metabolism in the GIT environment.

To reach the gut and colonise, the GIT microbiota must first pass through the stomach. The stomach is highly acidic due to the secretion of gastric acid and it is this property that makes the stomach inhabitable for most microbial species (136, 137), an exception being *Helicobacter pylori* which is highly adapted to acidic conditions (138). Different bacterial species residing in the GIT can grow at various pH values and this is an important factor in GIT colonisation (139). Additionally, in order for a bacterial strain to be considered a probiotic it must be able to survive the pH fluctuations that occur when it transits from the acidic stomach through the alkaline (due to bile secretion) environment of the upper part of the small intestine until it reaches the more or less pH-neutral large intestine (140).

The small intestine is more alkaline and aerobic when compared to the large intestine, and generally simple, mostly monomeric sugars are absorbed by the host here (13, 141). Microbes found in the small intestine generally metabolise simple sugars rather than larger and more complex, fibre-type glycans (142). Microbes that are present in the small intestine are therefore competing with the host for simple sugars, although secretion of anti-microbial peptides, IgA and bile help control bacterial growth in this gut location (143-145). Indeed, bacterial overgrowth in the small intestine may lead to host malnutrition (146). Bile fluid, which contains high bile salt levels, is produced by the liver and stored in the gall bladder before secretion into the small intestine (147). Prior to secretion primary bile acids, i.e. chenodeoxycholic acid and cholic acid, may become conjugated with either taurine or glycine (145, 148). These conjugated bile acids, also called bile salts, can then be further metabolised by certain bacterial components of the gut microbiota. For example, bile salt hydrolases are responsible for the deconjugation of the amino acids from bile acids (149, 150). Furthermore, these deconjugated bile acids may be converted into secondary bile acids by the GIT microbiota by 7 α-dehydroxylation, dehydrogenation and epimerization (145, 150) **(Fig. 1.1B)**. Bile acids are highly bactericidal due to their hydrophobic properties which promotes their insertion into the cell membrane of bacteria thereby causing cytoplasmic leakage (145). Therefore,

it is not surprising that bile acids have an impact on the GIT microbiota (151). Bile acids are generally reabsorbed in the small intestine, although they may escape re-adsorption and be present in the large intestine (147). Consequently, a bile salt gradient exists along the GIT ranging from approximately 40 mM to 0.5 mM with highest bile salt concentrations in the small intestine and lowest concentrations in the large intestine (151, 152). It should be noted that bile and bile salts have been shown to induce biofilm formation in certain gut commensals, most likely as a protective strategy to resist the deleterious effects of these compounds (153, 154), and this aspect will be dealt with in further detail below.

The large intestine contains the highest density and overall number of microbes, and represents the site where dietary fibre metabolism takes place (75). Fibre metabolism in the large intestine is important in dictating the microbial ecology of the large intestine by contributing to the relatively low luminal pH, that ranges from 5.5 to 7.5 (155), and by producing SCFA (see above). Nonetheless, a low carbohydrate and high protein diet may cause excessive protein fermentation in the large intestine, which may lead to the production of toxic and carcinogenic metabolites and increases the risk of pathogen proliferation and infection (156, 75). Therefore, dietary fibre metabolism is the sole or main carbon and energy source for specific microbes in the large intestine and is key to maintaining a healthy large intestine.

Plant glycans

Dietary fibres are derived from plant cell wall polysaccharides (157). The plant cell wall consists of a matrix comprising of cellulose fibrils, hemicellulose, pectin and lignin **(Figure 1.2)** (84). Hemicelluloses are polysaccharides with β-1,4-linked backbones of xylose, mannose or glucose, to form (arabino)xylan, mannan, and xyloglucan or β-glucan, respectively **(Figure 1.3)** (84, 158). Lignin is predominantly composed of dimerised phenolic compounds such as HCAs (158, 159). Pectin is composed of various highly variable polysaccharides including homogalacturonan (HG), xylogalacturonan, apiogalacturonan, rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII) (160). Pectic polysaccharides all contain an α-1,4-linked galacturonic acid backbone **(Figure**

1.4) (161). HG is the simplest pectic polysaccharide, consisting of unsubstituted α-1,4-linked galacturonic acid moieties, whilst RGI is associated with an α-1,4-linked, D-galacturonic acid and rhamnose-containing backbone which can be substituted by other polymers such as galactan, arabinogalactan and arabinan (162). RGI can also be decorated with phenolic compounds such as HCAs that cross-link through hydrogen bonding the oligosaccharide with other carbohydrate or lignin components (163). RGII is the most complex chain, with a HG backbone substituted with over 20 different glycosyl linkages and 5 different side chains (164). The various chemical bonds found in and enzymatic degradation of hemicelluloses and pectic polysaccharides are discussed in more detail below. Many of the complex, insoluble plant-derived dietary glycans that arrive in the large intestine are degraded by particular, so-called key stone species, Examples include *Bacteroides cellulosilyticus, Bacteroides caccae* and *Dysgonomonas gadei* species that have been identified as key stone species for the degradation of type II arabinogalactan due to their extracellular endo-β-1,3-galactanase activity (165). Following extracellular degradation of these carbohydrates, soluble oligosaccharides are released, which may then become available as metabolic substrates for other gut commensals, such as bifidobacteria. For example, *B. breve* UCC2003 can cross feed on certain GOS released from larch wood arabinogalactan by *Ba*. *cellulosilyticus* (166). Species/strains that can only degrade a narrow number of glycans or are dependent on other bacteria to provide such glycans are termed 'specialists' and may become extinct if these specific substrates do not become available in the gut (75, 92).

Metabolism of a particular glycan is typically regulated at the transcriptional level, where genes involved in the metabolism of such a glycan will only be transcribed in its presence with certain carbohydrates being preferentially metabolized over others (167). Different species or even different strains of a given species have evolved the ability to metabolise specific glycans. For instance, *Bacteroides ovatus* is able metabolise hemicelluloses, whilst *Ba*. *thetaiotaomicron* cannot utilize such saccharidic substrates (168). Strains belonging to *B. longum* subsp. *infantis* are typically capable of metabolising HMOs, whilst members of the closely related subspecies *B. longum* subsp. *longum* are not, yet are adapted to degrade certain plant glycans (169, 170). This division of resource utilization may be to avoid competition for carbon and energy sources between

species/strains. Furthermore, it should be noted that in the GIT microbes can live in microbial communities and mucosal layers, and that the presence of certain glycans in the gut can promote biofilm or microfilament formation (171, 172). Therefore, glycans represent a crucial factor in colonisation, persistence and survival of the gut microbiota in the large intestine.

1.3. Bifidobacterial survival in the gut environment

Bifidobacteria – general features.

Bifidobacteria are members of the Actinobacteria phylum harbouring genomes with a relatively high G + C content (considered approximately 50 % and higher) (173). They have been isolated from the GIT of many mammalian species, including humans, as well as of insects and birds (174). Bifidobacterial species are also present in the human oral cavity and abiotic environments such as sewage (173, 175). Bifidobacteria were first observed by Henri Tissier in the stools of healthy breast fed infants (176). Originally, bifidobacteria were taxonomically assigned to the lactic acid bacteria (LAB), being classified as *Lactobacillus bifidus* in Bergey's Manual of Determinative Bacteriology for much of the first half of the 20th century (177). Bifidobacteria employ a unique metabolic pathway for carbohydrate fermentation which is termed the fructose-6-phosphoketolase (F6PK) pathway or the 'Bifid Shunt' (177), which together with their distinctively high G + C content, above 50 %, justified their taxonomic classification as a genus from other LAB as was subsequently confirmed by 16S ribosomal RNA gene-based typing (178, 179). The first bifidobacterial genome sequence, i.e. that of *B. longum* subsp. *longum* NCC2705, was published in 2002 (180). Bifidobacterial genomes range from 1.7 Mb (*Bifidobacterium indicum*) to 3.16 MB (*Bifidobacterium scardovi*) (181, 182). Interestingly, genomes from bifidobacterial species isolated from the insect gut tend to be smaller than those from bifidobacteria isolated from the mammalian gut (183).

As mentioned above, the relative abundance of bifidobacteria has been shown to decrease following weaning and from adolescence into adulthood, when they are reported to be present at a relative abundance of between 3 % and 6 % (65, 66). For instance, bifidobacteria account for approximately 4 % relative abundance reported for adult

populations in Northern Europe (184). However, the prevalence of bifidobacteria in adult populations varies. Other studies, examining adult microbiomes from multiple countries have reported an enriched abundance of bifidobacteria in Japanese and Mongolian adult populations as compared to other adult populations (185, 186). However, it should be noted that the methods and procedures by which fecal samples were processed varied in each of these studies, which may have influenced the reported abundance values (187).

The bifidobacterial species that are present in the human gut may vary depending on host age. One study reported that the *B. longum* subsp. *longum* taxon is associated with both the adult and infant gut, whilst *B. breve* is more frequently associated with the infant gut (188). In contrast, another study reported that *B. longum* subsp. *longum* and *B. breve* were both associated with the adult and infant gut (189). *Bifidobacterium dentium* has been found to be in higher abundance in the elderly gut microbiota although its natural niche is believed to be the oral cavity (190). One reason to explain why particular species of bifidobacteria are more prevalent in the infant or adult gut may be that they are specialised to metabolise particular dietary carbohydrates. For example, *B. breve* and *Bifidobacterium kashiwanohense* are generally capable of metabolising HMOs as a consequence of breast-feeding (134, 191), whilst *B. longum* subsp. *longum* are generally specialised in the metabolism of plant fibres found in the adult diet (170, 180). The type of sample taken for microbiome analysis may therefore determine which bifidobacterial species are more likely to be detected. Some bifidobacterial species may be autochthons of the gut, for example *B. longum* subsp. *longum*, and are therefore capable of gut colonisation, whilst other species not isolated from the human gut, for example *B. animalis* subsp. *lactis* are allochthonous and are unlikely to be capable of (human) gut colonisation (43, 192, 193). Certain bifidobacterial strains or species, such as *B. longum* subsp. *longum*, are considered probiotic and are associated with various health benefits to the host, such as pathogen protection including production of acetate to protect against enteropathogenic infection (23), sequestration of iron at the detriment of gut pathogens (194), competing for epithelial binding sites with pathogens (194), immune modulation through exopolysaccharide production (EPS) (195), alleviation of IBS symptoms when supplied as a probiotic (196), and reducing the risk of contracting rotaviral diarrhoea

(197). On the other hand, just a single report has implicated bifidobacteria to cause bacteraemia in severely immunocompromised patients (198).

1.4. Plant-oligosaccharide utilisation by Bifidobacteria.

The bifid shunt – a unique carbohydrate metabolic pathway

As mentioned above, bifidobacteria possess a unique pathway for carbohydrate assimilation which is termed the F6PK pathway (177, 199). This complex pathway, with its key enzyme fructose-6-phosphoketolase, is very distinct from the homofermentation (Embden-Meyerhof-Parnas) or heterofermentative (phosphoketolase or pentose phosphate) glycolytic pathways (200, 201) and is exclusively found in the *Bifidobacteriaceae* family and members of the *Coriobacteriales* order (202-204). The F6PK pathway can convert both hexose and pentose sugars by fermentation into SCFAs (205), with a theoretically yield of 1.5 mol acetate and 1 mol of lactate for every mol of glucose consumed (177, 206), or a 1:1 ratio of lactate and acetate in the case of pentose sugar fermentation (203). However, the actual ratio of acetate to lactate produced depends on various factors including the individual strain, pH and growth rate, which in turn differs depending on the carbohydrate substrate utilised (203, 207, 208). High rates of sugar metabolism have been shown to produce more lactate, whereas slower rates of sugar consumption produce proportionally more acetic acid, formic acid and ethanol (209, 210). Bifidobacteria also produce millimolar amounts of succinic acid which was found to regenerate NAD^+ (209). The F6PK pathway theoretically produces 2.5 molecules of ATP per 1 metabolised glucose molecule, which is higher than the energy yield of homofermentation by lactobacilli species which yields 2 molecules of ATP per 1 molecule of glucose metabolised (203).

Carbohydrate import.

Bifidobacteria are capable of metabolising a diverse range of mono-, di-, and oligo-saccharides found in the GIT environment, which they mainly import into their

cytoplasm by means of ABC type transporters or major facilitator superfamily (MFS) transporters, such as proton symporters and proton-motive force-driven permeases (180, 211). Furthermore, most bifidobacterial species encode Phosphoenol pyruvate - Phosphotransferase systems (PEP-PTSs) (212, 213). Nonetheless, the number of encoded ABC transporters far exceeds that of other genome-specified carbohydrate transportation systems. For example, *B. longum* subsp. *longum* NCC2705 is predicted to encode 13 ABC type transporters, 3 MFS transporters, 1 PTS system, 1 glycoside pentoside cation symporter family transporter (GPH) and 1 major intrinsic protein family (MIP) transporter (214). Similarly, *Bifidobacterium longum* subsp. *infantis* ATCC15697 is predicted to encode 13 ABC transporter systems, including four ATP permeases, an ATPase and seven solute binding proteins (SBP) that are encoded by a 43 kb cluster dedicated to HMO utilisation (215). Carbohydrate-specific ABC transporters hydrolyse ATP in order to import carbohydrates against a chemical gradient (216). An ABC transport system typically consists of two transmembrane-associated proteins, which act as permeases to translocate the substrate across the membrane and two ATP-binding proteins that provide the energy required for transport (217). The nature of the internalized substrate, which can be a carbohydrate, peptide or other organic or inorganic molecules, of an ABC transporter system is determined by the fifth protein of the system, the so-called substrate binding protein (SBP), which binds the saccharidic substrate and presents it to the ABC transporter (217). SBPs specifically bind to a single substrate (or very related substrates) and this can affect growth rate of a strain on a less related substrate; for instance, the SBP of an ABC transporter specified by *B. animalis* subsp. *lactis* B1-04 binds preferentially to β-1,6-galactobiose over β-1,4-galactobiose, and this may in part contribute to faster growth of this strain on the former substrate (218). The heavy reliance on carbohydrate-specific ABC transporters by bifidobacteria for internalisation of their carbon and energy sources may reflect the need for members of this genus to be versatile in metabolising a diverse range of carbohydrates, including various oligosaccharides present in the gut (219, 220), rather than relying on PEP-PTSs, which are mainly restricted to monosaccharide utilisation (221). For example, an ABC transporter was found to confer the ability of *B. animalis* subsp. *lactis* B1-04 to metabolise the tri-saccharide raffinose (and related oligosaccharides) and this strain was able to outcompete *Ba*. *ovatus* when both strains are

co-cultured on raffinose (222). However, as a representative of its species *B. bifidum* PRL2010 is an exception and preferentially utilises PEP-PTS systems to import carbohydrates as this strain degrades complex carbohydrates extracellularly, thereby releasing mostly monosaccharides, explaining why PRL2010 encodes just two ABC transporters and four PEP-PTS systems (213).

Enzymatic degradation of plant-oligosaccharides by bifidobacteria.

A relatively high percentage, 13.7 %, of the *Bifidobacterium* genus pan-genome is dedicated to carbohydrate metabolism (174, 183), and a similar percentage, 13.23 % and 12.5 %, when representative genomes of *B. breve* and *B. longum* subsp. *longum*, respectively, are scrutinized (223, 224). However, when considering the *Bifidobacterium* core genome, just 5.5 % of these genus-wide conserved genes is dedicated to carbohydrate metabolic pathways suggesting that in order to survive in the GIT environment the acquisition of carbohydrate metabolic genes in the accessory genome is important (183). Bifidobacteria like other members the gut microbiota possess CAZymes dedicated to the breakdown of glycans in the GIT environment. Enzymes were originally categorised by the particular enzymatic reaction type they catalysed and were given enzyme commission (EC) numbers based on this (225). Enzymes can in addition be classified into CAZy families, where a CAZyme is assigned to be a member of a given family if it exhibits significant amino acid sequence similarity with the biochemically characterised founder member of that family, therefore taking into account protein fold and primary sequence similarity rather than just the type of enzymatic reaction (226). Therefore, an EC number can be distributed amongst several GH families and a single GH family may contain multiple EC numbers (225). Carbohydrate utilization profiles may aid in the subspecies classification of strains as *B. longum* subsp. *infantis* is specialised in HMO metabolism, whilst *B. longum* subsp. *longum* is dedicated to the metabolism of plant-derived glycans (224). Additionally, a strain-specific GH profile may help in categorising a strain into the correct species as GH families GH8, GH70, GH72, GH79 and GH94 were specifically found in subspecies *longum*, whilst GH34 and GH83 were only found in subspecies *infantis*; similarly unique EC numbers for subspecies *infantis* included EC 2.4.1.230 (kojibiose phosphorylase, GH65) and EC 3.2.1.18 (sialidase, GH33), whilst several EC

numbers were unique to the *longum* subspecies and included EC 2.4.1.4 (amylosucrase, GH13), EC 3.2.1.41 (pullulanase, GH13, GH49 and GH57), EC 3.2.1.45 (glucocerebrosidase, GH3, GH5, GH30 and GH116), EC 3.2.1.31 (β-glucuronidase, GH1, GH2, GH30, GH79 and GH154), EC 3.2.1.99 (arabinanase, GH43, GH49 and GH93), and EC 3.2.1.156 (reducing end xylose-releasing exo-oligoxylanase, GH8). Therefore, the enzymatic profile of a strain may aid taxonomic assignment (227). It must be noted that while these GH families are predicted to be encoded by the genomes of bifidobacterial species/strains examined in the above study, not all are currently affiliated with bifidobacteria in the CAZY database as such enzymes must first be purified and tested before they will be affiliated with a species/strain in the CAZY database.

Hydrolysis of a glycosidic linkage between two monosaccharides is mediated by two catalytic carboxylic residues in the corresponding GH, one being a proton donor represented by an acidic amino acid, while the other acting as a proton acceptor and represented by a basic amino acid **(Fig. 1.5)** (228). The process of hydrolysis can occur by two distinct routes, either (i) by means of a single displacement mechanism which takes place in a single step and which results in the inversion of the anomeric centre, or (ii) by a double displacement mechanism involving two catalytic steps resulting in the retention of the anomeric centre following hydrolysis (228, 229).

Inverting enzymes, involved in the single displacement mechanism, employ two catalytic amino acid residues in the catalytic site, typically glutamic or aspartic acid, that act as an acid and a base respectively, being typically 10 Å apart (228). The hydrolysis of a glycosidic bond in the single displacement mechanism begins with the protonation of the glycosidic oxygen by the acidic residue, whilst the basic residue activates a water molecule which then attacks and thereby hydrolyses the glycolytic bond (230). Retaining enzymes, which catalyse the double displacement mechanism, also have two catalytic residues that act as an acid and a base yet are approximately 5.5 Å apart (228). In the first step of the double displacement mechanism one residue initially protonates the glycosidic oxygen leading to the hydrolysis of the glycolytic bond and the formation of an oxocarbenium ion-like transition state. A glycosyl-enzyme intermediate is then formed by

the basic residue attacking the anomeric centre of the sugar thus concluding the first step. In the second step of the reaction, termed deglycosylation, the basic residue deprotonates a water molecule which in turn attacks the glycosyl-enzyme intermediate and results in the hydrolysis of glycosyl-enzyme intermediate (230). Enzymes that utilise the double displacement mechanism may under specific reaction conditions also catalyse the elongation of oligomers with new linkages, a process referred to as transglycosylation (229). For retaining enzymes if the nucleophile is instead a sugar molecule rather than a water molecule, for example under conditions of very high substrate concentration, transglycosylation may occur (228, 230, 231). Additionally, CAZymes can either degrade oligo- or polysaccharides at the end of the molecule, most commonly from the non-reducing end, or in between individual saccharidic moieties, activities that are referred to as exo or endo activity, respectively (232). The remainder of this review will focus on bifidobacterial GHs and CEs that are known to be involved in plant-oligosaccharide degradation.

Xylan and xylo-oligosaccharides (XOS).

Bifidobacteria are capable of growth on several plant-derived poly/oligo-saccharides and their derived monomers (207, 208). Specifically, the *B. longum* subsp. *longum* taxon seems to be particularly well adapted to plant-based carbohydrate utilisation (224). Hemicelluloses include carbohydrates that generally possess a β-1,4-linked backbone, for example xylan, which is composed of β-1,4-linked D-xylose moieties (158). Furthermore, this xylan backbone can be decorated or substituted with L- or D-arabinose, xylose, galactose and galacturonic acid (233). Based on the nature of these substituents xylan is further categorised into arabinoxylan (AX), glucoronoxylans (GX) and glucoronoarabinoxylans (GAX) (234). AX from corn may also contain α-1,2-linked galactose to arabinose side chains (235, 236) **(Figure 1.3)**.In order to obtain access to the xylan/XOS backbone bifidobacteria must first remove the arabinose, xylose, galactose, HCA and other substitutions attached to the xylan or XOS backbone.

Multiple enzymes are needed to degrade the xylan backbone. Xylanases or endo-1,4-β-xylanases (EC 3.2.1.8, GH5, GH8, GH10, GH11, GH30, GH51 and GH98) are

endo-acting enzymes that hydrolyse the internal β-1,4 bond between D-xylose residues within a xylan polymer to produce XOS (with a degree of polymerisation of between two and nine) **(Figure 1.6A)** (237). Currently, no bifidobacterial strain/species is known to be able to grow on the large insoluble xylan backbone. Therefore, it is likely that in the GIT species such as *Ba. ovatus*, *Ba. xylanisolvens* or *Ba. intestinalis* degrade the xylan backbone into soluble XOS, which then becomes available for other species to utilise (238-240). Species such as *B. longum* subsp. *longum* and *Bifidobacterium adolescentis*, are able to metabolise xylan-derived XOS (241, 242) and several enzymes have been implicated in the degradation of this oligomeric substrate by bifidobacteria. β-D-xylosidases (EC 3.2.1.7, GH1, GH2, GH3, GH43, GH51, GH52, GH54, GH116 and GH120) are exo-enzymes which can hydrolyse XOS starting at the non-reducing xylose residue. For instance, a β-1,4 xylosidase (EC 3.2.1.37) (GH51) from *B. breve* K-110 was shown to elicit activity against *p*-Nitrophenyl (*p*Np) β-D-xylopyranoside, yet was shown to elicit very limited activity against xylan (243). Furthermore, *B. adolescentis* LMG10502 encodes two β-xylosidases: XylB (GH120) which hydrolyses XOS but not xylobiose, and XylC (GH43), which hydrolyses xylobiose (244) **(Figure 1.6B)**. In addition, the GH8 RexA or reducing-end, xylose-releasing exo-oligoxylanase enzyme (EC 3.2.1.156)(245) from *Bifidobacterium adolescentis* LMG10502 was shown to elicit limited activity against xylan, no activity against xylobiose or *p*Np-β-D-xylopyranoside, though was shown to exhibit activity against XOS with a DP of 3 and above **(Figure 1.6C)** (246).

Transcriptional and proteome analysis of *B. animalis* subsp. *lactis* BB-12 grown on XOS revealed expression of a number of xylanases, β-xylosidases and ABC transporters (246). Bifidobacterial species/strains that are able to utilise XOS, such as *B. longum* subsp. *longum* and *B. adolescentis*, may only be able to metabolize XOS up to a degree of polymerisation (DP) of six, i.e. xylohexose due to size limitations of the corresponding XOS transport system (247, 248). It must also be noted that generally bifidobacterial CAZymes are intracellular although extracellular hydrolysis of XOS by an apparently extracellular bifidobacterial β-1,4-xylosidase has been reported for *B. adolescentis* (248).

AX, AXOS, arabinan, arabinogalactan and corn GAX.

The xylose residues in xylan and XOS can be mono-substituted with L-arabinose at the C(O)2 or C(O)3 positions or di-substituted with L-arabinose at both C(O)2 and C(O)3 positions, while these arabinose substitutions can either be α-1,2-linked or α-1,3-linked (158, 249). Only a limited number of bifidobacterial species/strains, e.g. *B. longum* subsp. *longum*, are able to metabolize such arabinoxylan (AX) and arabinoxylo-oligosaccharide (AXOS) glycans (109, 224, 250). Depending on the particular bifidobacterial species/strain different components of AX or AXOS are utilised. One study has grouped bifidobacterial species/strains into five groups depending on if and what AX, AXOS or XOS components are being metabolised: cluster I, metabolism of monosaccharides arabinose and xylose, but no metabolism of XOS or arabinose substituents; cluster II, metabolism of mono- or di-substituted arabinose, yet no utilisation of the XOS backbone; cluster III, utilisation of the XOS backbone but no utilisation of arabinose substituents; cluster IV, utilisation of both arabinose substituents and XOS, up to xylotetraose of AXOS; cluster V, utilisation of AXOS including up to xylohexaose XOS chains (251). Therefore, the presence of AX, AXOS and XOS in the GIT supports growth of various bifidobacterial species/strains either directly or indirectly through possible cross-feeding activities (249). In this sense, *Ba. ovatus* has been shown to support growth of *B. adolescentis* when they interact on simple xylans, such as wheat AX and birch glucoronoxylan (Rogowski et al., 2015). However, *Ba. ovatus* cannot cross-feed with *Bifidobacterium* sp. when they use complex dietary xylans, such as corn AX. The reason is that *Bifidobacterium* lacks the catalytic apparatus needed to metabolize the oligosaccharides released by *Ba. ovatus* into the media. This is consistent with the fact that *B. adolescentis* is unable to metabolise corn AX, even if it is pretreated with the glycoside hydrolases located in the surface of *Ba. ovatus* (Rogowski et al., 2015).

Pectin is composed of multiple complex glycans that can be utilised by the gut microbiota (252, 253). Probably because of its complexity there are currently no known

bifidobacterial species that are able to directly metabolize pectin **(Figure 1.4)**. It is therefore presumed that other gut commensals such as *Ba. thetaiotaomicron* degrade these large polymers extracellularly and that certain bifidobacterial species can then scavenge the released mono- and oligosaccharides, as shown previously by co-cultivation of *B. longum* subsp. *longum* with *Ba. thetaiotaomicron* in the presence of arabinogalactan (254). *B. longum* subsp. *longum* strains have been shown to grow on the pectic components arabinan and arabinogalactan (255, 256). Arabinan consists of an α-1,5-linked L-arabinose backbone that can be mono- or di-substituted with either α-1,2-linked and/or α-1,3-linked L-arabinose (161). Type I arabinogalactan is usually linked to other pectin-associated glycans, whereas type II arabinogalactan is O-linked to a protein backbone. Both arabinogalactan types are key components of the plant cell wall (257, 258). Type I arabinogalactan is composed of a β-1,4-linked D-galactose backbone substituted by α-1,5-linked L-arabinose, while type II arabinogalactan is composed of a β-1,3-linked D-galactose backbone that can be substituted with α-1,3-linked arabinose and α-1,6-linked galactose side chains (161, 165, 258).

α-L-arabinofuranosidases (EC 3.2.1.55, GH1, GH2, GH3, GH5, GH39, GH43, GH51, GH54 and GH62) are exo-acting enzymes that can cleave arabinose moieties from the polymeric backbone of xylan, XOS, galactan or arabino-oligosaccharides (AOS) (259, 260). Arabinofuranosidases typically remove mono-substituted α-1,2-linked and/or α-1,3-linked arabinose from their particular substrate backbone (261, 262), although certain arabinofuranosidases are specialised in removing arabinose from a di-substituted substrate (262). The ability to degrade AXOS has been shown to be species/strain dependent and some bifidobacterial species/ strains are only able to metabolise the arabinose substitutions on XOS (251). An α-arabinofuranosidase (GH51) produced by *B. longum* subsp. *longum* has been shown to release arabinose from AX (260), while AbfA (GH43) from *B. adolescentis* was shown to remove arabinose residues from the C(O)2 and C(O)3 positions of mono substituted xylose, and AbfB (GH51) and AXHd3 (GH43) were demonstrated to release arabinose residues from the C(O)3 of disubstituted xylose residues (262, 263). L-arabinofuranosidases can also act as exo-enzymes on arabino-oligosaccharides present in arabinan or arabinogalactan; an α-L-arabinofuranosidase (GH1) from *B. adolescentis* was shown to possess exo-activity on α-1,5-linked arabino-

oligosaccharides (DP 2-5) (264). Similarly, the *B. longum* subsp. *longum* ArafC (GH43) was shown to be capable of removing α-1,2-linked and α-1,3-linked arabinose side chains of AX and arabinan, yet ArafD (GH43) was shown to exhibit hydrolytic activity towards α-1,5-linked arabinan (255). α-L-arabinofuranosidases can also release arabinose side chains from galactose residues in arabinogalacatan; BlArafA (GH43) an α arabinofuranosidase produced by *B. longum* subsp. *longum* can release α-1,3-linked arabinose from β-1,6-GOS (265). Endo-α-arabinases (EC 3.2.1.99) hydrolyse the α-1,5-linkage within the arabinan backbone (266) and it is likely that arabinofuranosidases must remove the L-arabinose substituents before the backbone can be effectively cleaved. β-L-arabinofuranosidases (EC 3.2.1.185, EC 3.2.1.185, GH127, GH142 and GH146) remove β–linked arabinose substitutions from plant-oligosaccharides; β-linkages are less common and found on type II arabinogalactan linked to plant cell wall proteins (267). In *B. longum* subsp. *longum*, β-arabinofuranosidases HypBA1 (GH127) and HypBA2 (GH121) release arabinose from β-1,2-linked arabinosaccharides (DP 2-3) linked to hyproxyline (268, 269). Several bifidobacterial α-L-arabinofuranosidases and β-L-arabinofuranosidases have been reported in literature and their salient features are summarised in Table 1.1.

Various enzymes are required to degrade plant-derived galactan. Exo-acting β-1,3-galactanases (EC 3.2.1.145) cleave the β-1,3-D-galactose backbone of arabinogalactan even in the presence of β-1,6-D galactose side chains through an by-pass mechanism (165, 270). Exo-acting β-1,4-galactanases (no designated EC number) cleave terminal β-1,4-linked galactose bonds (258). An exo-β-1,3 galactanase, (GH43 subfamily 24) (Bl1,3Gal) isolated from *B. longum* subsp. *longum* was shown to hydrolyse β-1,3-linked galacto-oligosaccharides (DP between 2 and 5), de-arabinosylated larchwood arabinogalactan (271). This Bl1,3Gal enzyme was unusual as it had a higher activity for β-1,3-galactan when the latter substrate was substituted with β-1,6-side chains, apparently recognizing these side chains as a specificy determinant in the active site. Similarly, BgaA (GH2) of *B. breve* UCC2003 was shown to cleave β-1,3-linked galactobiose/triose (166) **(Fig. 1.7A)**. An exo-β-1,6-galactobiohydrolase (Bl1,6Gal, GH30) from the same species was shown to degrade β-1,6 linked galactose (DP between 2 and 4) and β-1,6-galactan but was not able to degrade arabinose substituted substrates (265) **(Figure 1.7B)**. Furthermore, depending on the linkage type of the galactan backbone degradation may

involve endo-acting β-1,3-galactanases (EC 3.2.1.181, GH30) (258), β-1,4 galactanases (EC 3.2.1.89, GH53) (272) or β-1,6-galactanases (EC 3.2.1.164, GH30) (258).

In *B. longum* subsp. *longum*, an extracellular endo-acting β-galactanase, GalA, was found to be capable of cleaving β-1,4 and β-1,3-galactan linkages (273) **(Figure 1.7C)**. The extracellular GalA (GH52) homolog in *B. breve* UCC2003, which is present in certain strains of this species, was found to elicit hydrolytic activity towards galactan releasing GOS (274). GalA (GH52) is found in a galactan utilisation cluster in both *B. breve* UCC2003 and *B. longum* subsp. *longum* strains; this galactan utilisation cluster that encodes GalA, an ABC type transporter, and GalG (GH42), a β-galactosidase; was shown to be upregulated when this *B. breve* UCC2003 is cultivated on galactan and GOS as its sole carbon sources (256, 274). β-galactosidases (EC 3.2.1.23, GH1, GH2, GH35, GH39, GH42, GH59, GH147 and GH165) hydrolyse linkages between a galactose moiety and another sugar moiety and several β-galactosidases have been identified in *B. bifidum*, *B. longum* subsp. *longum*, *B. longum* subsp. *infantis* and *B. breve*, being able to hydrolyse β-1,3, β-1,4 or β-1,6 linkages in GOS and HMO substrates (134, 275-278) **(Figure 1.7D)**.

Finally, the backbone or side chains of these plant-derived oligomers may also be substituted with HCAs. Most hemicelluloses and pectic plant polymers also have HCAs attached by an ester bond to the (O) 5 position of the sugar moiety (158, 279). HCA-specific esterases (EC 3.1.1.73, CE1 and CE6) catalyse the hydrolysis of an ester bond between a HCA, for example ferulic acid and *p*-coumaric acid, and a sugar moiety (arabinose, galactose or xylose) on AX and pectin plant-oligomers (280). Esterases have an alpha/beta hydrolase fold, a consensus motif (Gly-X-Ser-X-Gly) and a catalytic triad consisting of Ser-His-Asp residues (281). Bifidobacterial esterases active against HCAs have been described, including the CaeA esterase, whose encoding gene is located within the same genetic locus as the genes encoding GH enzymes that are predicted to be involved in AOS utilisation (113, 114, 282).

Regulation of carbohydrate metabolism

Carbon catabolite repression (CCR) refers to a global regulatory mechanism by which bacteria can preferentially metabolise the 'optimal' carbon source that has the greatest energy yield, amongst a mixture of carbon sources, and involves inhibition of the metabolic pathways of the less preferred carbon sources (283). This is important in the GIT environment where potentially multiple carbohydrate sources are present and the optimal carbon source must be consumed to increase chances of survival in the gut. There are many mechanisms of CCR and this can vary from species to species. For instance, CCR may involve transcriptional activation, transcriptional down regulation and translational regulation (284). In the CCR paradigm, many bacteria, such as *Escherichia coli* the 'optimal' substrate glucose is metabolised preferentially (285), whereas for *B. longum* subsp. *longum* and *Streptococcus thermophilus* lactose is preferentially metabolised over glucose (286-288). The preference of other sugars over glucose for metabolism is also termed reverse CCR (284). CCR-resembling regulation has previously been described in bifidobacteria. In particular, in *B. breve* UCC2003 a FOS utilisation cluster inducible by growth on sucrose or Actilight, a commercial FOS prebiotic, was shown to be downregulated in the presence of glucose and/or fructose - sucrose mixes (289). CCR may be important from an ecological perspective, as it may avoid species/strain competition for limited carbon sources in the gut environment (290). However, CCR is not the only model to describe the regulation of carbohydrate metabolism in bacteria. Indeed, *B. breve* and *Corynebacterium glutamicum*, both members of the *Actinobacteria* phylum, have been shown to globally regulate their central metabolic flux and control co-metabolism of multiple sugars (291, 292).

In order to control gene expression, bacteria commonly employ transcription factors (TFs), which typically bind to a specific DNA sequence, termed an operator, close to a promoter sequence where it can activate or repress transcription activity initiated from that promoter (293). TFs generally possess a helix-turn-helix (HTH) motif which allow them to recognize the operator and a companion domain that can bind to ligands or interact with other proteins thereby controlling the HTH domain and consequently its DNA binding ability (294, 295). TFs are categorised into different families based on sequence

similarity, examples being represented by the well characterised LysR, OmpR, LacI/GalR, and AraC/XylS families (296). LacI-type TFs for example have a HTH DNA binding domain at their N-terminus, a core domain to bind sugar ligands and a multi-merisation domain for the formation of dimers and/or tetramers (297). LacI-type TFs generally act as repressors, though they can also increase transcription of their target promoters (293). With regards to carbohydrate metabolism, the LacI-type TF typically acts to only allow expression of a given set of genes if the corresponding carbohydrate is present in the growth medium. For instance in *E. coli*, the cytoplasmic presence of allolactose, which indicates the presence of lactose in the environment, prevents binding of the LacI repressor to the *lac* operator sequence, thereby allowing the RNA polymerase access to the *lac* promoter region, and activating transcription of the lactose (*lac*) operon (298, 299). Similarly, LacI-type TFs in bifidobacteria typically act as carbohydrate-specific transcriptional repressors and are therefore important allowing only appropriate expression of carbohydrate metabolism genes in the presence of the corresponding saccharidic substrate in the GIT environment.

LacI-type transcriptional regulators are the most prevalent and abundant family of bifidobacterial TFs; in one study they were shown to account for 63 % of all identified regulators encoded by ten bifidobacterial genomes (300). TFs have been shown, *in silico* and *in vitro*, at a local level to control genes and/or operons involved in carbohydrate metabolism for various carbohydrates including HMOs (301), galactan (256), melezitose (302), AOS (303), FOS (289), ribose (304) and cellodextrin (305). Nonetheless, other types of TFs have been reported to be involved in transcriptional control of genes involved in carbohydrate metabolism. Examples are represented by a GntR-type TF for sialic acid utilisation (306), a so-called repressor open reading frame kinase (or ROK) TF for raffinose and stachyose metabolism (302), and a NagC/XylR-type repressor involved in sulfated sugar metabolism regulation (307) **(Figure 1.8)**.

Central carbohydrate metabolism in bifidobacteria is represented by the 'Bifid Shunt', which is regulated by two LacI-type regulators, designated AraQ and MalR1 (292), employing a mechanism that is reminiscent to that reported for *C. glutamicum* (291). This mechanism of global carbohydrate regulation may be of advantage to

bifidobacteria in the GIT environment allowing these gut commensals to quickly and effectively respond to the various different types and structurally diverse glycans that can be present in the GIT at any given time.

1.5. Bifidobacterial survival in response to bile.

In the GIT environment, bifidobacteria also encounter bile acids and bile salts and due to the bactericidal properties of these compounds bifidobacteria have developed various strategies to deal with this imposed bile stress. Bile salt/acids target the bacterial cell membrane and exposure of bifidobacteria to bile acids reduces internal pH, disrupts the transmembrane potential and results in leakage of cytoplasmic contents (308). Growth of bifidobacteria in the presence of bile has been reported to result in loss of certain glycolipids from the cell membrane and a decrease in surface hydrophobicity (309). Conversely, porcine bile has been shown to increase bifidobacterial cell surface hydrophobicity (154). Similarly, in *B. animalis* subsp. *lactis* IPLA 4549 and a bile-adapted derivative, exposure to bile was shown to cause a decrease in membrane fluidity, changes in membrane fatty acid composition and cell surface protein content, a decrease in phospholipid ratios, and distortion of the cell surface including formation of membrane vesicles (310). Changes in the expression of fatty acid synthesis, generally downregulation, in several different species/strains of bifidobacteria, some of which were bile adapted, have been reported in response to bile exposure (311-313). The presence of an extracellular polysaccharide (EPS) layer around a bacterial cell may exert protective effects against bile stress. In *B. animalis* subsp. *lactis* bile exposure has been shown to induce EPS production, while EPS was also shown to elicit a protective effect against a porcine bile challenge in *B. breve* and bile salt exposure in *B. breve* and *B. longum* subsp. *longum* and *B. pseudocatenulatum* (195, 314, 315). The cell surface proteome is also altered upon exposure to bile. Expression of DnaK, which can act as a plasminogen receptor, and an enolase, was found to be upregulated in response to bile exposure and was hypothesised to facilitate colonisation of *B. animalis* subsp. *lactis* (316). Genes encoding oligopeptide uptake were also shown to be transcriptionally upregulated in *B.*

breve and *B. longum* subsp. *longum* in response to bile and oligopeptide uptake in the presence of bile has been demonstrated for *B. longum* subsp. *longum* (313, 317).

Many bifidobacterial species/strains, such as human isolates belonging to *B. longum* subsp. *longum* and *B. breve*, are known to encode a bile salt hydrolase (BSH) which can remove the taurine- or glycine amino group from internalized bile salts to release deconjugated bile salts (318-323). Bacteria lacking BSH activity are therefore believed to be more susceptible to the bactericidal effects of bile acids/salts (324). BSH activity produced by certain bifidobacteria has been reported to detoxify bile acids, provide nutrients through release of the amino groups, alter the cell membrane by incorporating bile salts, and overall contribute to the persistence of such microbes in the gut environment (325). However, the exact mechanism by which bile acid deconjugation activity aids in bifidobacterial survival has yet to be elucidated. Certain bifidobacterial species possess efflux pumps to extrude bile acids/salts from their cytoplasm/membrane. The *ctr* gene in *B. longum* subsp. *longum* NCIMB 702259 encodes a cholate efflux transporter in the sodium/bile acid family of transporters, *B. longum* subsp. *longum* NCC2705 and *B. breve* UCC2003 specify major facilitator superfamily (MFS) transporters that confer resistance against bile/bile salts, while *B. breve* UCC2003 additionally produces ABC transporters that confer resistance to sodium cholate (313, 326, 327).

When bile salts cross the cell membrane, often derived from bile acids deconjugated by extracellular BSH activity, they cause the acidification of the cytoplasm and adaption to bile stress also increases resistance to acid stress indicative of cross over between bile and acid stress adaption (328). In order to counteract cytoplasmic acidification due to bile acid exposure, *B. animalis* has been shown to possess a bile-inducible F_1F_0 ATPase, which extrudes protons at the expense of ATP (while it can also produce ATP by allowing protons to flow into the cytoplasm) (329). Carbohydrate metabolism is also affected when bifidobacteria are exposed to bile. Bile-adapted strains of bifidobacteria have been shown to preferentially utilise maltose or glucose in contrast to the wildtype strain (330). In another study bile-adapted strains were shown to express different glycosyl hydrolase activities against synthetic substrates: for instance, one bile-

adapted *B. longum* strain was shown to exhibit increased arabinofuranosidase activity against *p*Np-arafuranosidase (328). Therefore, bile appears to influence carbohydrate metabolism and this may be due to increased metabolic/energy requirements when coping with bile stress. Central metabolism is also influenced by bile, and an increase of the activity of xylulose 5-P/fructose 6-P phosphoketolase, which is a key enzyme in the bifid shunt, has been reported in both *B. longum* subsp. *longum* and *B. animalis* subsp. *lactis* (311, 331). It should be noted that various studies have been carried out with different concentrations of bile and different types of crude bile (porcine or bovine) or bile salts, and this is likely to cause variable responses to bile stress reported in literature. It has also been reported that biofilm in bifidobacteria is induced by bile and bile salts (154). Therefore, bifidobacteria have adapted a robust and multifaceted response to bile stress involving cell surface modification, altered carbohydrate metabolism and biofilm formation in order to survive in the GIT environment.

1.6. Biofilm formation by bifidobacteria.

Biofilm formation describes a process that occurs when micro-organisms live together in microbial communities either attached to a surface or in flocs, called micro-filaments and are enclosed by an extracellular matrix (332, 333). It has been shown that many gut commensals can form biofilms in the GIT environment (153, 334). Therefore, it is likely that biofilm formation is a key strategy to survive and persist in the GIT environment. Biofilm formation is a complex and multi-step process. The initial stage of biofilm formation called attachment involves micro-organisms attaching to a surface either through non-specific electrostatic interactions caused by the physical properties of the surface, or by specific release of extracellular DNA (eDNA) which promotes further electrostatic interactions between the micro-organisms and the surface, or by the expression of cell wall-associated proteins that bind to ligands coated on the surface (335-337). The next biofilm formation phase is the accumulation stage which involves protein-protein interactions between cell wall associated proteins and/or ligands and secretion of an extracellular matrix (EM) (335, 337). The EM can be composed of protein, carbohydrates, such as EPS, and/or eDNA (338). This is then followed by the maturation

stage, during which further development of the biofilm structure takes place through continued EM secretion. Finally, the dispersal stage involves cells in a biofilm detaching in order to return to planktonic growth. Dispersal can involve DNases to cleave DNA in the EM (339), proteins with surfactant properties such as phenol-soluble modulins (340) and enzymes that degrade the carbohydrate component of the EM, such as Dispersion B which degrades the β-1,4-glycosidic bonds in the *N*-acetyl glucosamine-containing EM (341). Biofilms are often formed in response to environmental stress, such as acid stress, antibiotics, bile stress or nutrient starvation, in order to increase microbial survival (342-344). Biofilm formation is commonly regulated by a process called quorum sensing; a cell density-dependent signalling system (345). One of the best characterised systems for quorum sensing-controlled biofilm formation present in a wide range of bacteria is that involving auto-inducer-2 (AI-2) (345). The AI-2 molecule is produced by the intracellularly located LuxS, a S-ribosylhomocysteinase, and released in the growth medium, where its concentration is sensed by a two component signaling receptor, which at a particular threshold AI-2 concentration will trigger biofilm formation (as well as other adaptive responses) (346) **(Figure 1.9)**.

Bifidobacteria have been reported to form biofilm and microfilaments *in* vitro when induced by bile and bile salts (by exposure to 0.5 % taurocholic acid or 5 % porcine bile) and in the gut environment (154, 172). The AI-2 quorum sensing system may be involved in bifidobacterial biofilm formation. It has previously been reported that transcription of the *luxS* gene is upregulated upon exposure of *B. breve* UCC2003 to bile (313). AI-2 production has been reported for several bifidobacterial species/strains and overexpression of LuxS from *B. longum* susbp. *longum* NCC2705 has been shown to induce and increase biofilm formation (347-349). However, there does not seem to be a complete AI-2 system in bifidobacteria as they appear to lack the typical AI-2 sensors LuxP and/or LsrB (349, 350). Currently, besides these studies little is known about biofilm formation in bifidobacteria. Therefore, more work is needed to ascertain what the molecular mechanisms of biofilm are in bifidobacteria and whether biofilm is important in the survival and/or persistence of bifidobacteria in the GIT environment.

1.7. Discussion

The GIT environment is a dynamic, highly competitive and challenging ecological niche for bacteria to colonise. The physical environment of the gut itself is diverse and subjects microbes to low acid conditions in the stomach, high concentrations of bile in the small intestine and microbial competition from the densest microbial population in the human body which is located in the large intestine. To further complicate matters, the diet of the human host changes as we age moving from breast milk in infancy to complex glycan fibres in adult hood. Therefore, in order to survive the GIT environment bifidobacteria must overcome acid stress, bile stress, be able to metabolise complex carbohydrates and be able to respond appropriately both metabolically and physiologically to the ever-changing conditions of the GIT environment. Bifidobacteria represent a key genus among the gut microbiota and are present in the gut throughout life from infancy, adolescence, adulthood to old age. They are seen as a general indicator of health due to their purported probiotic properties. Therefore, understanding how human-specific members of this genus colonize, persist and survive in the GIT environment is crucial if we wish to harness their health-promoting capabilities. It is clear that the highly flexible and energy efficient carbohydrate metabolism of bifidobacteria gives these microbes an advantage in the GIT and enables them to metabolise a range of different carbohydrates from HMOs in breast milk to complex plant glycans in the adult diet. Specialisation of different species of bifidobacteria on specific carbohydrates is believed to allow this genus to occupy many metabolic niches in the GIT whilst avoiding niche competition among the various members of the *Bifidobacterium* genus. Furthermore, its apparently unique regulatory control of carbohydrate metabolism allows this genus to be flexible and efficient in its carbohydrate metabolism whilst in the gut. Furthermore, bifidobacteria have a diverse set of mechanisms to deal with bile acid exposure, representing a major bactericidal challenge in the GIT by, among others, modulating carbohydrate metabolism, bile salt efflux and biofilm formation. Bifidobacteria therefore seem to have developed a multi-faceted arsenal of approaches to survive the various challenges of the GIT environment.

1.8. Author Contributions

S.M.K., J.M.M and D.v.S. wrote and edited the manuscript.

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1.10. Tables and Figures

Table 1.1. Summary of characterised bifidobacterial arabinofuranosidases.

Enzyme Name/ classification	Substrates	GH family	Reference	Species
AbfB α-L-arabinofuranosidase	Arabinan, AX, arabinobiose - arabinopentose	GH51	(260)	*B. ll*
BXA43 α-L-arabinofuranosidase	XOS (DP 2-4) *p*NP α L araf *p*Np β D Xyl	GH43	(351)	*B. al*
BAD0156 α-L-arabinofuranosidase	pNP α L araf α 1,5 arabinosaccharides	GH1	(264)	*B. a*
BlArafC α-L-arabinofuranosidase	*p*NP α L araf Arabinan AX	GH43	(255)	*B. ll*
BlArafD α-L-arabinofuranosidase	*p*NP α L araf Arabinan	GH43	(255)	*B. ll*
BlArafA α-L-arabinofuranosidase	*p*NP α L araf α-1,3 Ara*f* Gal$_3$ Ara*f*-α-1,3-Ara*f*-α-OMe Radish AG Larch AG Arabinan	GH43	(265)	*B. ll*
Blon_0625 α-L-arabinofuranosidase	*p*NP α L araf	GH3	(352)	*B. li*
HypBA2 β-L-arabinofuranosidase	β1,2 Arabiose β linked arabinotriose - hyproxyline Arabinan Debranched Arabinan	GH121	(269)	*B. ll*
HyBA1 β-L-arabinofuranosidase	β 1,2 linked Arabinose – hyproxyline (DP 2 and 3) Arabinobiose - ME	GH127	(268, 353, 354)	*B. ll*
AfuB-H1 α-L-arabinofuranosidase	*p*NP α L araf	GH51	(355)	*B. ll*
AbfA α-L-arabinofuranosidase	AX AXOS *p*NP α L araf *p*NP β Xyl	GH43	(356)	*B. a*

Table legend: L arf : L arabinofuranose, Xyl: D-xylopyranoside, ME: methyl group, Gal; galactose, OMe; o linked methyl group, AG; arabinogalactan, AX; Arabinoxylan; AXOS: arabinoxylo-oligosaccahrides; *B. ll*; *Bifidobacterium longum* subsp. *longum*, *B. al; Bifidobacterium animalis* subsp. *lactis, B.b; Bifidobacterium breve, B. a; Bifidobacterium adolescentis, B. li; Bifidobacterium longum* subsp. *infantis*

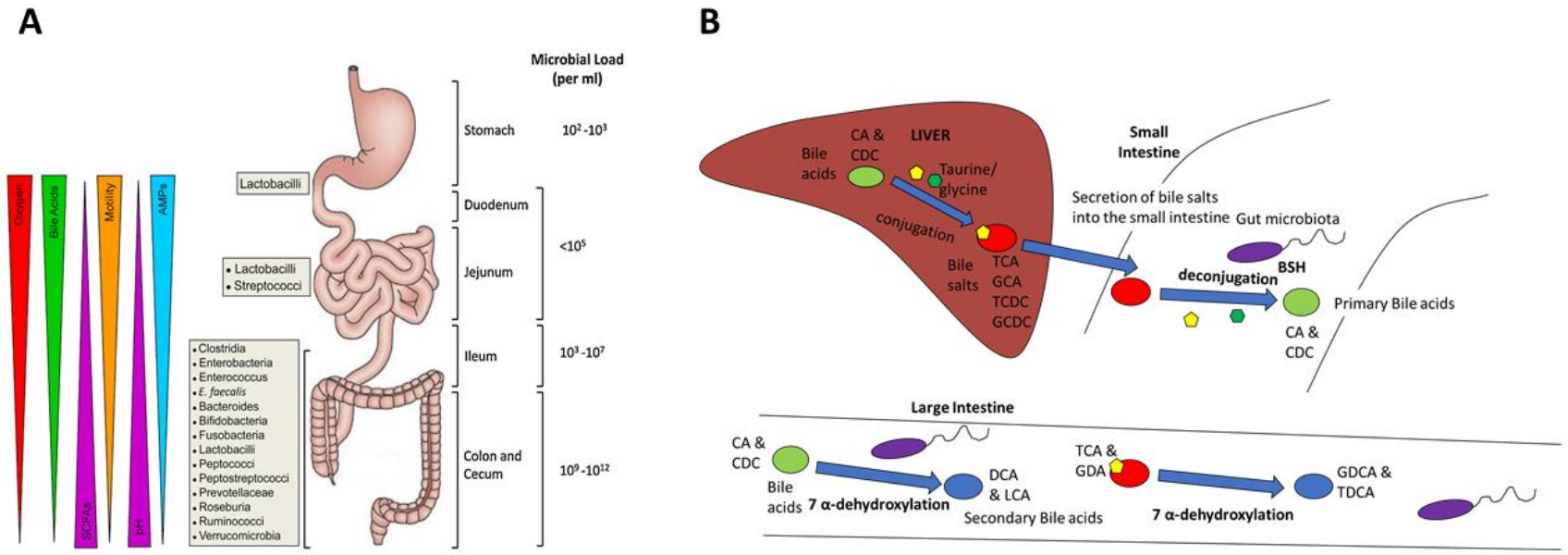

Figure 1.1. Summary of the gut physical environment and bile acid/salt bioconversion by the gut microbiota.

Different compartments of the gastrointestinal tract harbour different physical conditions e.g. oxygen, pH, bile acids, small chain fatty acids (SCFAs), anti-microbial peptides (AMPs) and transit time (motility) all vary. A summary of the various physical conditions along the gastrointestinal tract adapted from Reinoso Webb *et al.* (357) **(A)**. A summary of some of the bile acid/bile salt conversions by the gut microbiota **(B)**. Primary bile acids such as cholic acid (CA) and chenodeoxycholic acid (CDC) are conjugated in the liver with a taurine or glycine amino acid, thus becoming bile salts Taurocholic acid (TCA), Glycocholic acid (GCA), taurochenodeoxycholic acid (TCDC) and Glycochenodeoxycholic acid (GCDC). Bile salts are then secreted into the small intestine. The gut microbiota can then act on these bile salts with a bile salt hydrolase enzyme to deconjugate bile salts back into primary unconjugated bile acids. Most bile acids/salts are re-absorbed in the small intestine however some bile acids and salts escape readsoprtion and are also present in the large intestine. Further modifications of bile salts by the gut microbiota include 7-α-dehydroxylation which converts CA and CDA into deoxycholic acid (DCA) and lithocholic acid (LCA). Bile salts, TCA and GDA can also be converted into Glycodeoxycholic acid (GDCA) and Taurodeoxycholic acid (TDCA).

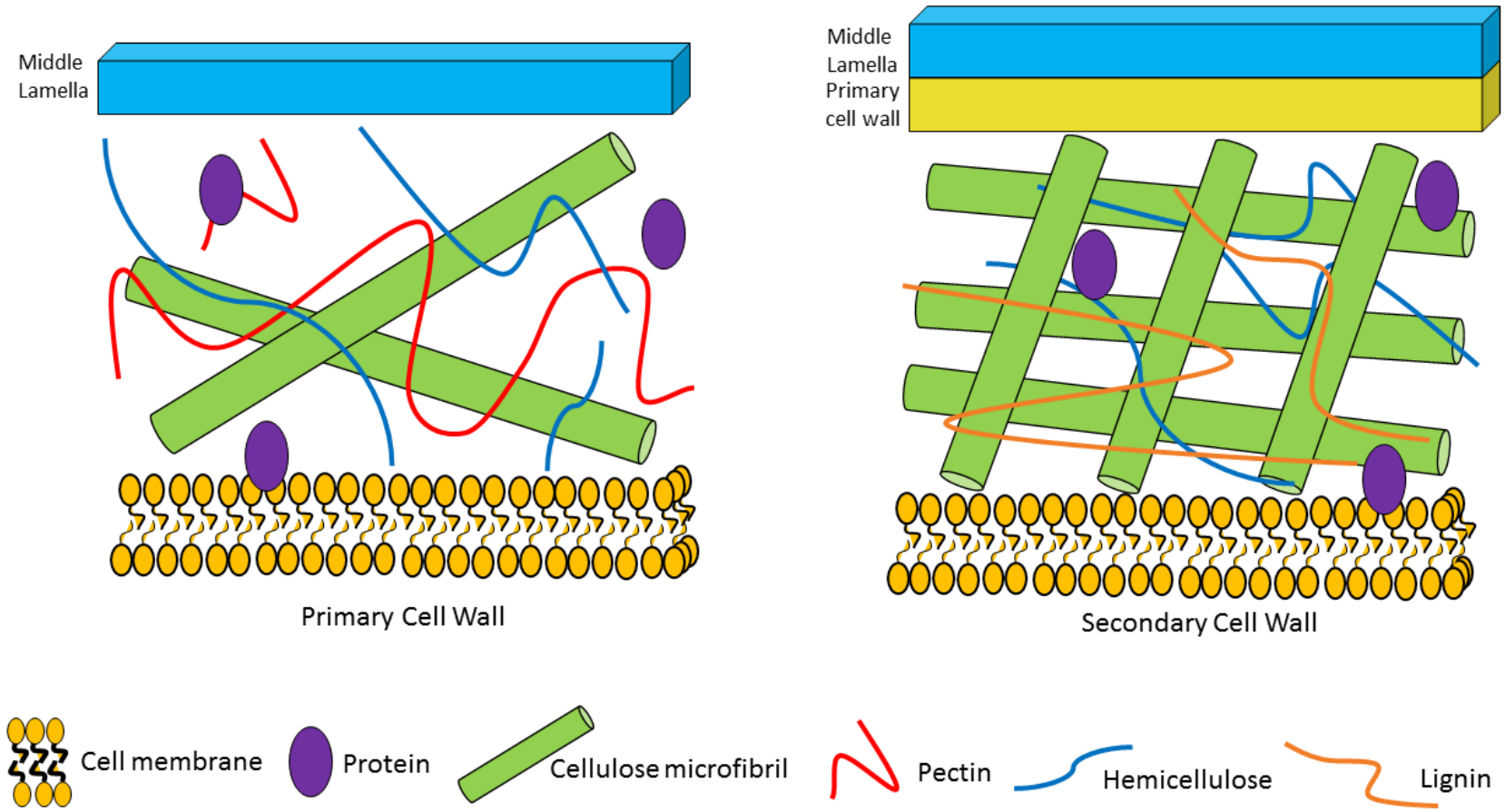

Figure 1.2. Plant cell wall composition and associated plant oligosaccharides.

The primary cell wall is composed of cellulose microfibrils, hemicellulose and pectin. The secondary cell wall contains cellulose microfibrils, hemicellulose and lignin.

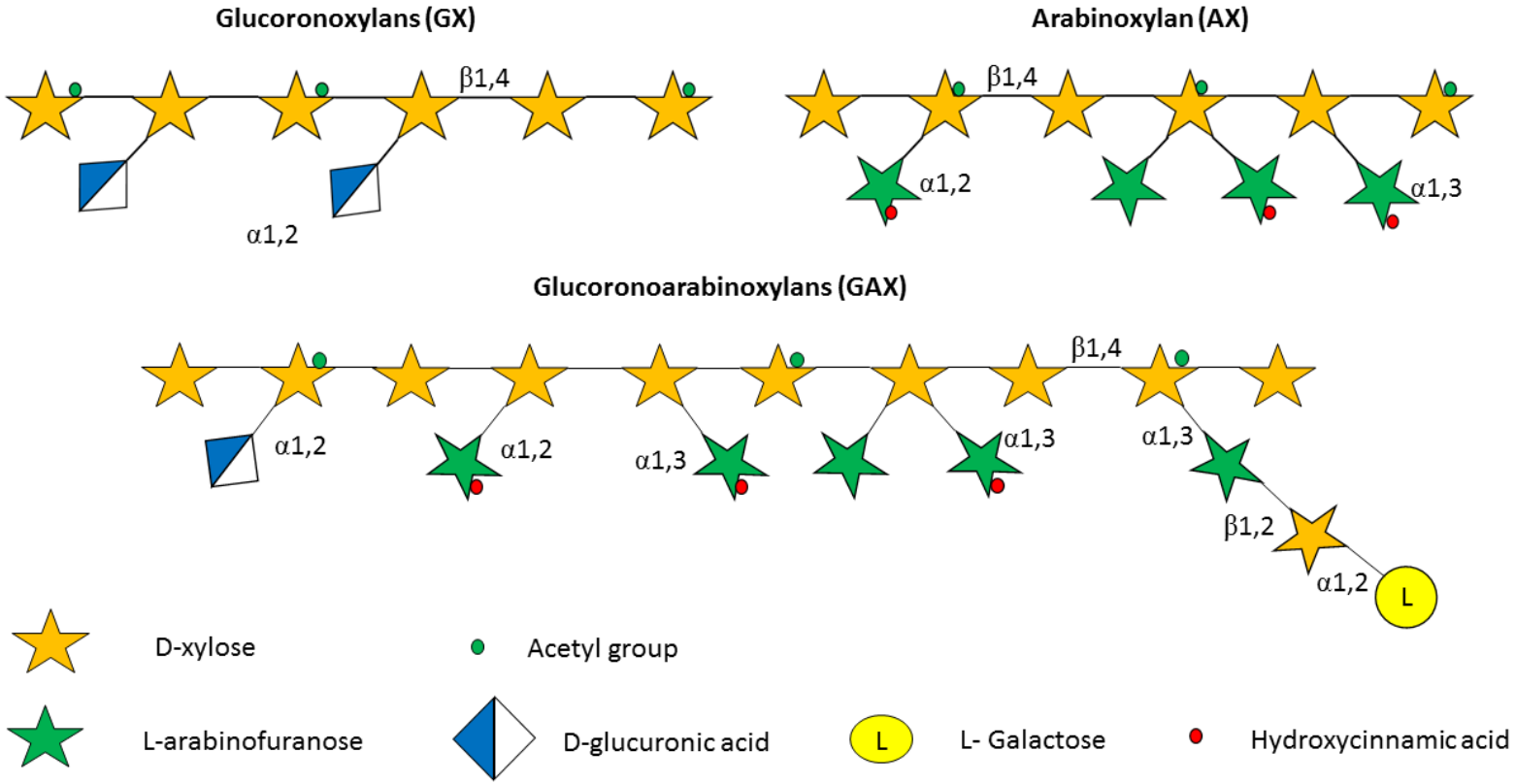

Figure 1.3. Structure of hemicelluloses found in the plant cell wall.

Hemicelluloses consist of a β-1,4-linked D-xylose backbone (xylan) that is acetyl group substituted. Glucoronoxylans (GX) the xylose backbone is substituted with D - glucuronic acid. Arabinoxylans (AX) have α-1,2-linked and α-1,3-linked arabinofuranose substitutions. The backbone can be mono- or di-substituted. Finally, glucoronoarabinoxylans (GAX) possess the same backbone and arabinose substitutions as AX, yet with additional D - glucuronic acid moieties that are α-1,2-linked to the backbone, as well as D-xylose and L-galactose moieties that are β-1,2-linked and α-1,2-linked, respectively, to the arabinose substitutions.

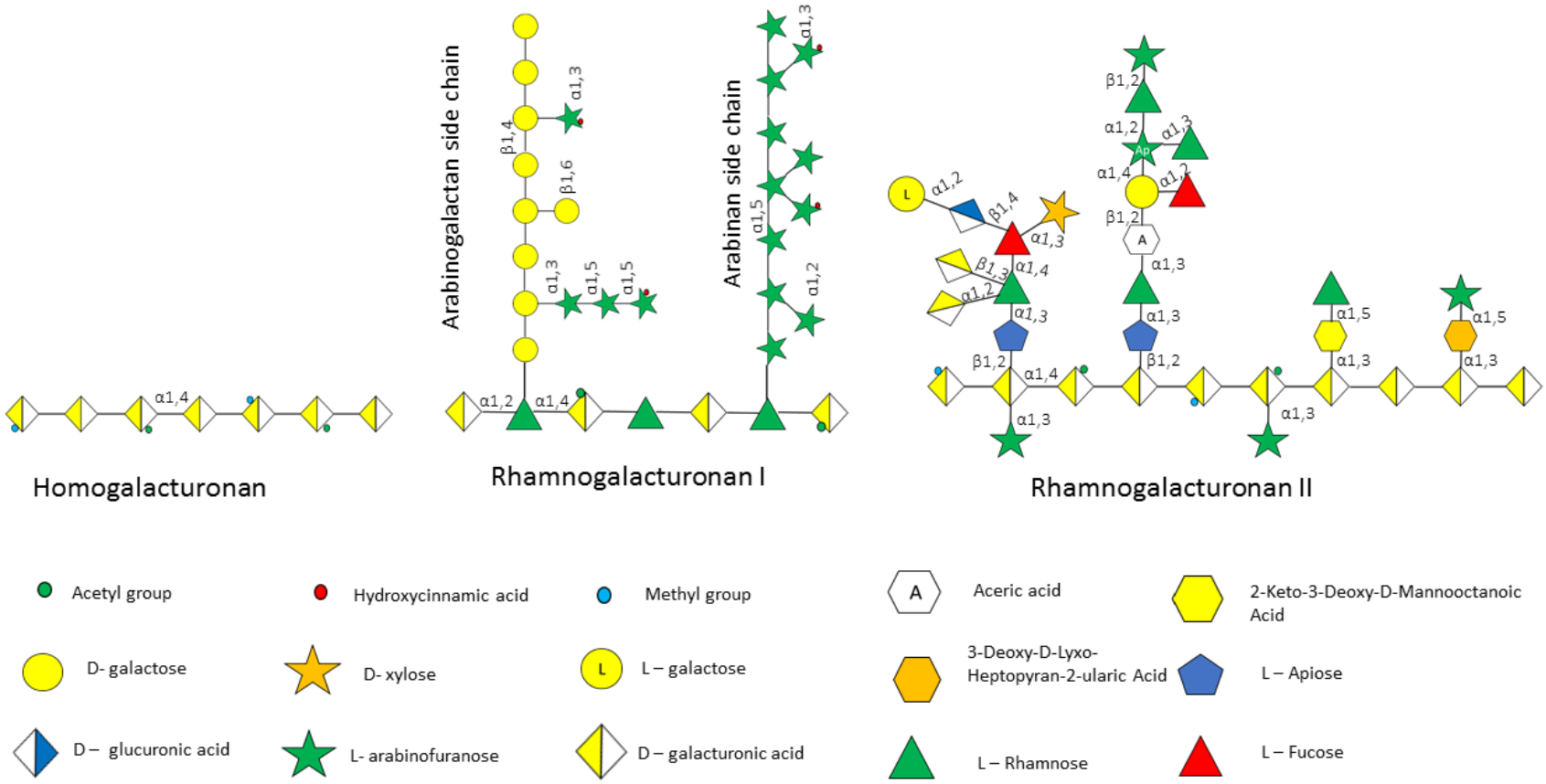

Figure 1.4. Pectin polysaccharides associated with the plant cell wall.

Pectin is made up of several polysaccharide domains including homogalacturonan, rhamnogalacturonan I and Rhamnogalacturonan II.

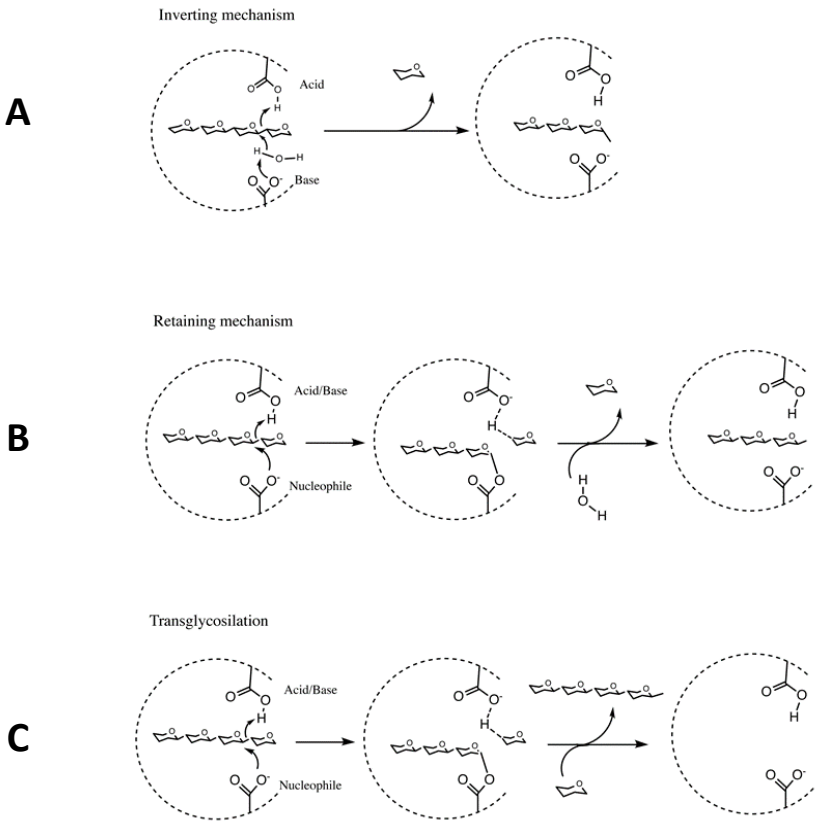

Figure 1.5. Summary of inverting hydrolysis, retaining hydrolysis and transglycosylation.

Summary of inverting single displacement mechanism **(A)**. Summary of retaining double displacement mechanism **(B)**. Summary of transglycosylation (C). See text for details of the reactions.

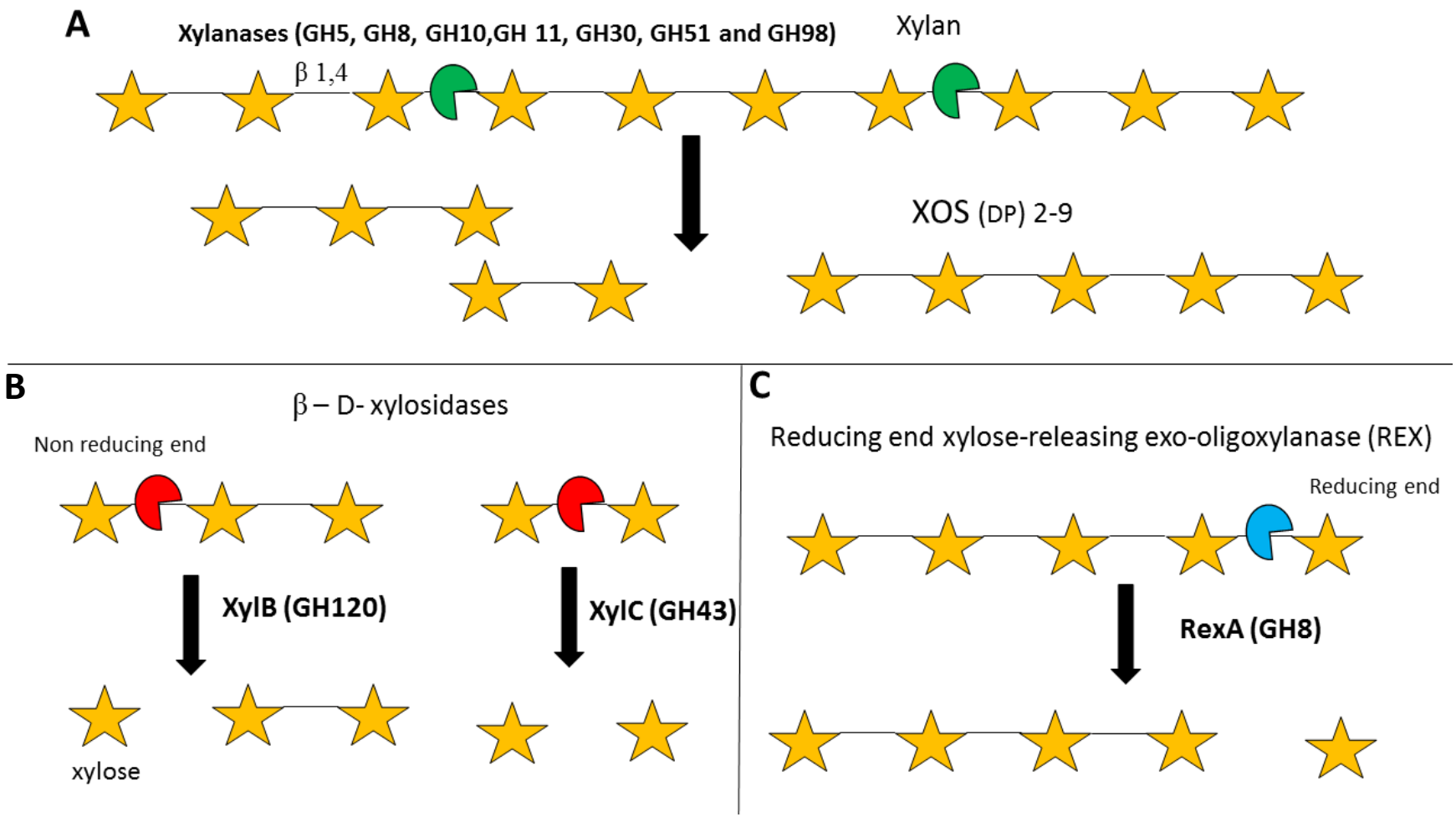

Figure 1.6. Enzymatic degradation of xylan and XOS.

Degradation of the xylan backbone to XOS by xylanases (A). Degradation of XOS by β –D- xylosidases (B). Degradation of XOS by a 'Reducing end xylose releasing exo-oligoxylanase (C). See text for details. DP = degree of polymerization. Enzyme names are indicated in bold.

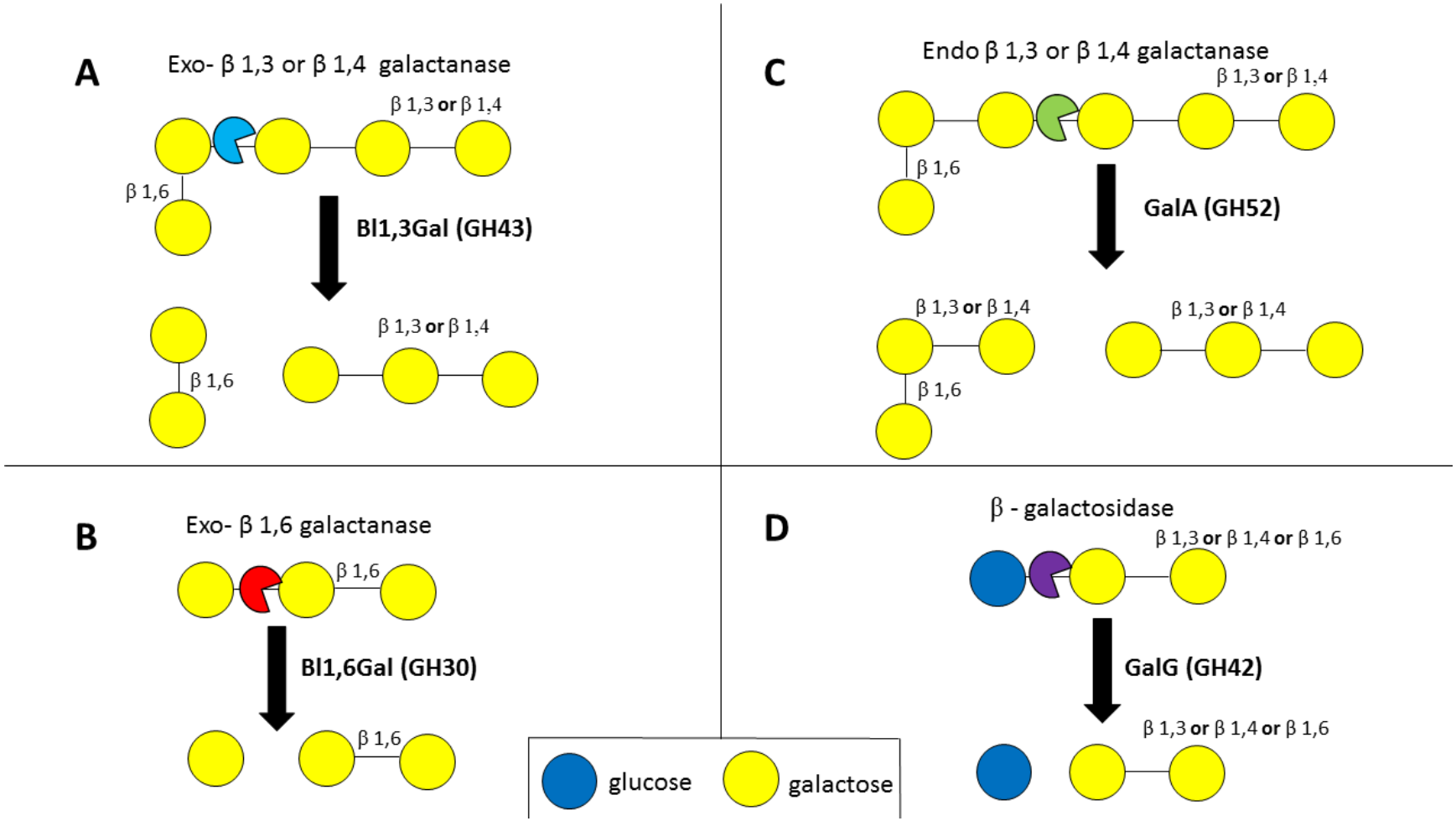

Figure 1.7. Enzymatic degradation of galactan.

Degradation of the galacatan by exo- β 1,3 or β 1,4 galactanases **(A)**. Degradation of galactan by exo - β 1,6 galactanases **(B)**. Degradation of the galacatan by endo β 1,3 or β 1,4 galactanases **(C)**. Degradation of a galactose- sugar moiety bond by β –galactosidases **(D)**. Enzyme names indicated in bold.

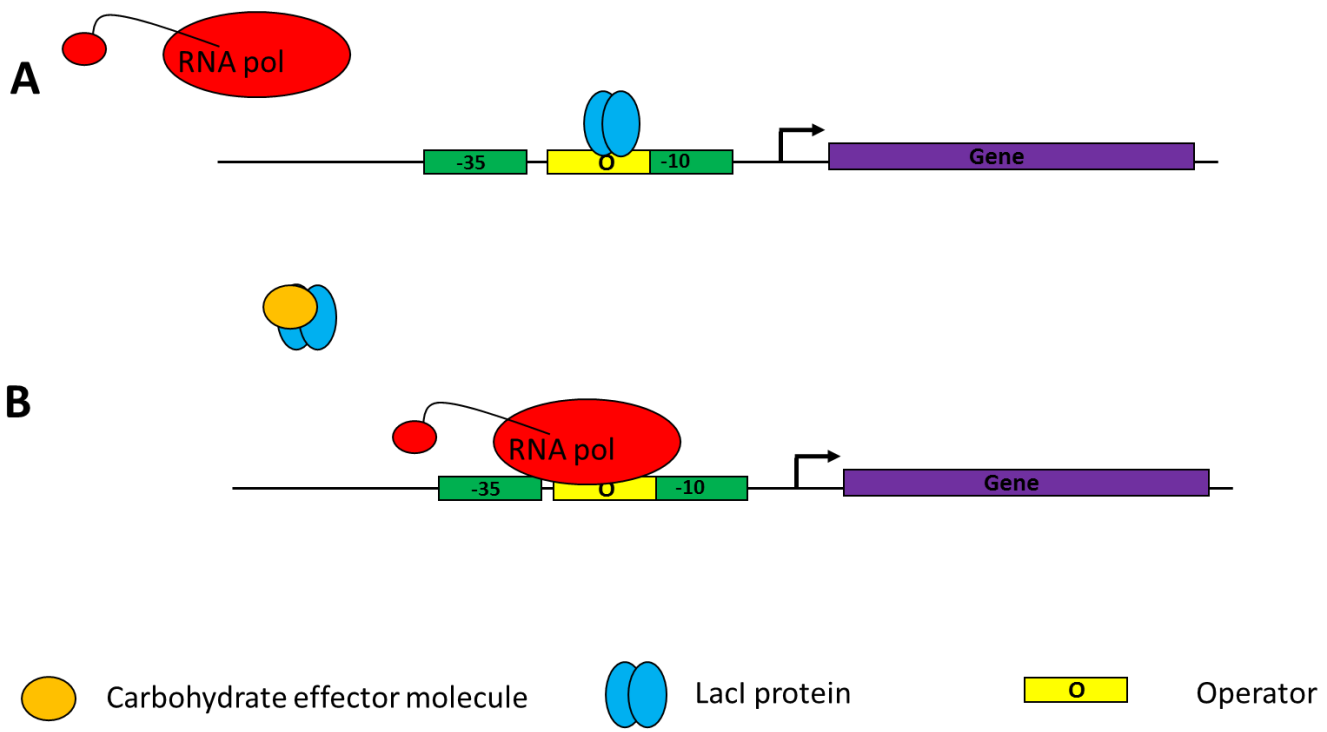

Figure 1.8. General schematic of transcriptional repression by a LacI-type repressor.

In the absence of the sugar effector molecule the LacI-type repressor binds the operator sequence, and blocks access of the RNA polymerase to the promoter region or prevents transcriptional progression of the RNA polymerase, thereby inhibiting transcription **(A).** When a sugar substrate is metabolized and enters the cell this substrate or a metabolic derivative will act as an effector molecule by binding to the LacI-type repressor protein changing its conformation in a manner that prevents the LacI to bind to the operator sequence (typically by preventing dimerization of the LacI-type repressor). Therefore, the RNA polymerase is free to recognize the promoter region and initiate and progress transcription of the gene **(B)**.

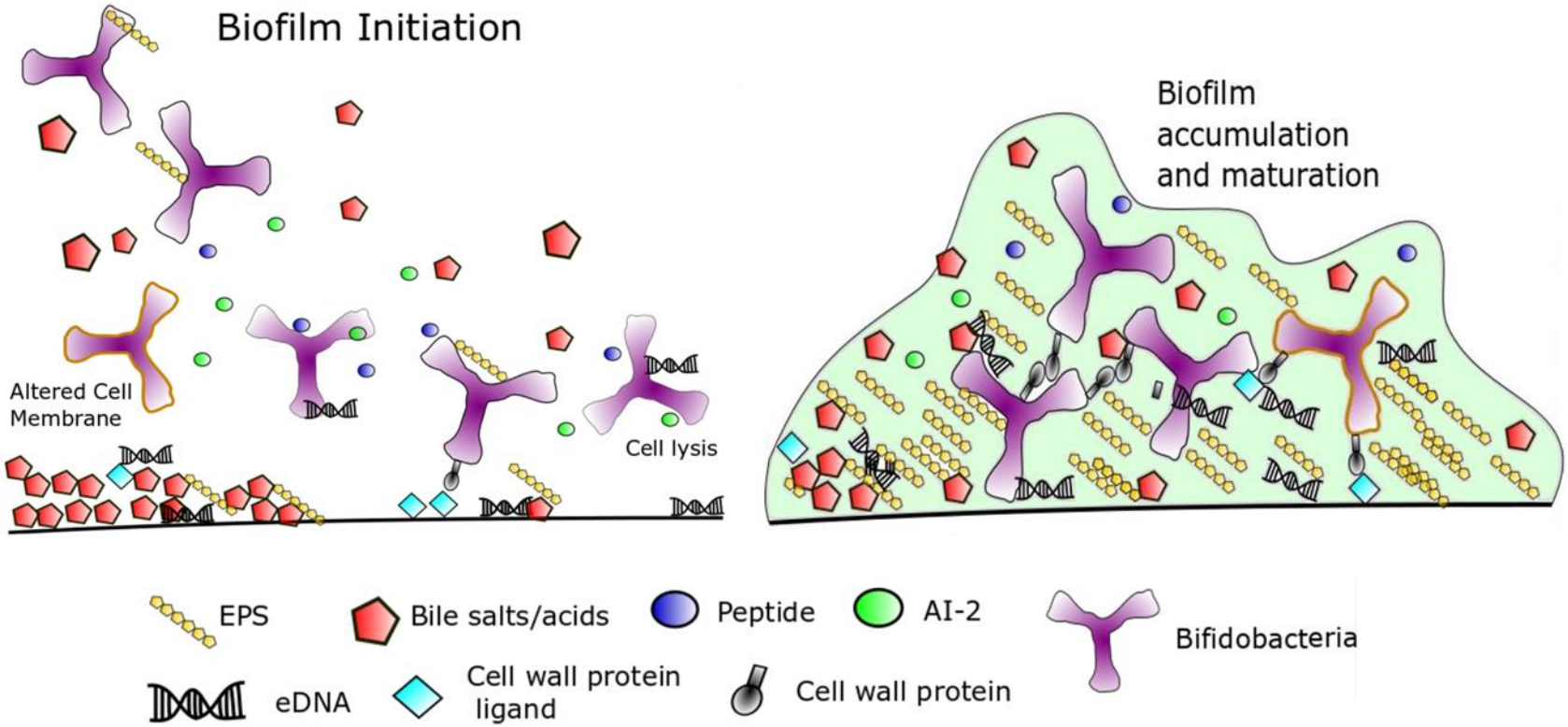

Figure 1.9. The stages of biofilm formation.

See text for details of biofilm formation.

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Chapter II

Characterisation of a Hydroxycinnamic acid Esterase from the *Bifidobacterium longum* subsp. *longum* taxon.

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2.1. Abstract

Bifidobacterium longum subsp. *longum*, a common member of the human gut microbiota with perceived positive health effects, is capable of metabolising certain complex, plant-derived carbohydrates which are commonly found in the (adult) human diet. These plant glycans may be employed to favourably modulate the microbial communities in the intestine. Hydroxycinnamic acids (HCAs) are plant phenolic compounds, which are attached to glycans, and which are associated with anti-oxidant and other beneficial properties. However, very little information is available regarding metabolism of HCA-containing glycans by bifidobacteria. In the current study, a gene encoding a HCA esterase was found to be conserved across the *B. longum* subsp. *longum* taxon and was present in a conserved locus associated with plant carbohydrate utilisation. The esterase was shown to be active against various HCA-containing substrates and was biochemically characterised in terms of substrate preference, and pH and temperature optima of the enzyme. This novel HCA esterase is presumed to be responsible for the release of HCAs from plant-based dietary sources, a process that may have benefits for the gut environment and thus host health.

2.2. Introduction

Bifidobacteria are Gram-positive gut commensals of various mammals, insects and birds, where their presence is associated with a number of beneficial effects (1). Such beneficial effects include pathogen inhibition (2, 3), immune modulation (4, 5), reduction in the symptoms of irritable bowel syndrome (6) and cholesterol reduction (7). In humans, bifidobacteria are particularly abundant and prevalent in the infant gut, though their relative abundance reduces upon weaning and upon ageing of their host (8). Members of the *Bifidobacterium* genus commonly metabolise a range of dietary and host-derived carbohydrates, with the precise substrate nature of this versatile carbohydrate metabolism being strain/species specific (9). The ability to metabolise diet- and host-derived carbohydrates is believed to enable efficient bifidobacterial gut colonisation and persistence. For example, infant-associated bifidobacterial species/strains can typically metabolise human milk oligosaccharides (HMO) present in breast milk, while species/strains found in adults tend to metabolise various dietary plant polysaccharides (10-13).

Members of the *Bifidobacterium longum* subsp. *longum* taxon have the capacity to metabolise various plant cell wall polysaccharides, such as arabinoxylan, and pectic components, such as arabinan (14-21). Therefore, these polymeric glycans are considered suitable substrates to stimulate growth of *B. longum* subsp. *longum* in the adult gut. Plant phenolic compounds, such as ferulic acid and *p*–coumaric acid, also sometimes referred to as hydroxycinnamic acids (HCAs), may be present as substitutes to the L-arabinose moieties of such plant polymers by means of ester linkages (22, 23). Although various studies have described aspects of arabinoxylan and arabinan metabolism, little is known about HCA metabolism by bifidobacteria.

HCAs are commonly found in various foods, being present in cereals, fruit, vegetables and coffee, among others (24, 25). HCAs have been associated with a variety of beneficial effects, including diabetes resistance in rats (26), intestinal pathogen inhibition (27), inhibition of platelet aggregation (28), anti-oxidant and anti-carcinogenic activities (29). Several studies have shown that certain fungi possess HCA esterases with broad substrate specificity, while more recently it has been demonstrated that bacterial

species, including lactobacilli and bifidobacteria, produce esterases that cleave artificial HCA-containing substrates and are (presumed to be) capable of removing HCAs from plant substrates (30-35). Therefore, gut commensals that produce HCA-active esterases are believed to play a role in releasing HCAs from plant carbohydrates. It may be that HCAs must be first removed from the plant carbohydrate to give access to other enzymes involved in plant carbohydrate degradation. The purpose of HCA release may also provide an energy advantage to bacteria as it has been shown HCAs can be used as external electron acceptors (36, 37). Furthermore, HCAs can inhibit growth of spoilage bacteria in high concentrations and HCA metabolism is thought to detoxify inhibitory HCAs (38).

Esterases and lipases are two important groups within the hydrolase class of enzymes. Both esterases and lipases cleave ester bonds, possess an α/β hydrolase fold and generally exhibit a consensus sequence of (Gly-X–Ser–X–Gly) around the catalytic triad residues Ser–His–Asp (39, 40). Esterases, in contrast to lipases, generally follow Michaelis-Menten kinetics and hydrolyse substrates that are less than six carbons in length (39, 41). Esterases can also be categorised into four groups based on substrate preference (42).

In the current study we identified a hydroxycinnamoyl acid esterase-encoding gene, designated *caeA*, in the genome of members of the *B. longum* subsp. *longum* taxon, positioned within a conserved locus predicted to be associated with arabinoxylan and arabinan metabolism. Heterologous expression, purification and subsequent characterization of the CaeA protein demonstrated that it indeed represents a genuine esterase, as opposed to a lipase, and can cleave several HCA-containing substrates. The biochemical properties of the CaeA esterase were investigated and the optimal enzyme pH and temperature ascertained. Therefore, this hydroxycinnaminic esterase is another *B. longum* subsp. *longum* enzyme that may contribute to this taxon's ability to metabolise plant-derived polysaccharides.

2.3. Materials and Methods

Bacterial strains, plasmids, growth conditions and chemicals. Bacterial strains and plasmids used in this study are summarised in Table 1. Bifidobacteria were routinely cultured on Reinforced Clostridium Agar (RCA) or in modified deMan, Rogosa, Sharpe medium (mMRS) supplemented with 1 % (w/v) lactose (Sigma-Aldrich, Steinheim, Germany) and 0.05 % (w/v) cysteine–HCL (Sigma-Aldrich) (43). All bifidobacteria were cultivated under anaerobic conditions in a modular atmosphere-controlled system (Davidson and Hardy, Belfast, United Kingdom). *Lactococcus lactis* strains were grown in M17 broth (Oxoid, Basingstoke, Hampshire, United Kingdom) supplemented with 0.5 % (w/v) glucose at 30°C. Where required media was supplemented with 5 μg ml^{-1} chloramphenicol. For RCA ethyl ferulate plate assays, RCA medium was supplemented with 0.1 % (v/v) ethyl ferulate dissolved in 96 % ethanol. Methyl ferulate, ethyl ferulate, methyl *p*–coumaric acid, methyl sinapinate, methyl caffeic acid (caffeate) and feruloyl glucose were all dissolved in 96 % ethanol (Carbon Chemicals, Ringaskiddy, Ireland) and sourced from Carbosynth, Berkshire, United Kingdom. Para-nitrophenol (*p*-Np) acetate, *p*-Np butyrate, *p*-Np octanoate and *p*-Np dodecanoate were purchased from Sigma–Aldrich. All ions were purchased from Sigma-Aldrich.

Nucleotide sequence analysis. Bacterial genomes were assessed using the Artemis genome browser (44) employing the annotated genome of *B. longum* subsp. *longum* NCIMB 8809 genome (20). Nucleotide analysis was completed using the programs SeqMan and SeqBuilder of DNASTAR software (DNASTAR, Madison, WI, USA). Annotation of protein function and HMM-HMM homology detection, both under standard settings, were determined using BlastP and HHPred, respectively (45-48). Protein alignments were generated using Clustal omega (49).

DNA manipulations. All DNA manipulations were carried out as previously described (50). Chromosomal DNA was isolated from *B. longum* subsp. *longum* NCIMB 8809 using the GenElute Bacterial Genomic DNA Kit (Sigma–Aldrich). Primers for genomic amplifications were synthesised by Eurofins (Ebersburg, Germany). Genomic PCR reactions were performed with Q5 High-Fidelity 2X Master Mix (New England Biolabs, Herefordshire, United Kingdom) or Taq PCR master mix (Qiagen GmbH, Hilden,

Germany). PCR products were cleaned using the Roche High Pure PCR Kit (Roche Diagnostics, Basel, Switzerland). Plasmid DNA was isolated using the High Pure Plasmid Prep Kit (Roche Diagnostics) with an added initial step of incubating resuspended cells with 30 mg ml^{-1} lysozyme (Sigma-Aldrich) at 37°C for 30 min. Restriction enzymes (Roche Diagnostics) and T4 (Promega) were used as per manufacturer's instructions. Colony PCR was performed using Extensor Hi-Fidelity PCR Master mix (Thermo Fisher Scientific, Waltham, United States). DNA electroporation procedures for *B. breve* and *L. lactis* were as previously described (51, 52). The integrity of all constructs was confirmed by DNA sequencing (performed by Eurofins, Ebersburg, Germany).

Plasmid construction for heterologous expression of *caeA* in *B. breve* UCC2003. To construct the pNZ44caeA overexpression plasmid, the gene encoding the cinnamoyl esterase (B8809_1755), designated here as *caeA*, was amplified from the genomic DNA of *B. longum* subsp. *longum* NCIMB 8809 as a template using the polymerase Taq PCR master mix and primers CaeAF and CaeAR. Primer sequences used in this study are listed in Table 2. The generated PCR amplicon was restricted using NcoI and XbaI, and ligated to pNZ44 that had been similarly restricted with NcoI and XbaI. The resulting plasmid construct was electroporated into *L. lactis* NZ9000 and transformants were selected for by Cm^R resistance. Transformants containing the desired recombinant plasmid were confirmed by colony PCR using Extensor PCR Master Mix. Plasmid DNA was extracted from such transformants and clone integrity was confirmed by sequencing, resulting in plasmid pNZ44caeA, which was then electroporated into *B. breve* UCC2003 using chloramphenicol selection.

Ethyl ferulate plate assay. The ethyl ferulate plate assay was carried out as described previously with modifications (53). Bacterial cultures were grown in mMRS supplemented with 1 % (w/v) lactose overnight and were spot plated (10 µl) on to RCA with 0.1 % (v/v) ethyl ferulate. Plates were then incubated anaerobically for 72 hours at 37°C. A zone of clearing on the RCA ethyl ferulate plate around the colonies was taken as an indication of esterase activity.

Expression and purification of CaeA in *L. lactis* NZ9000. To construct the pNZcaeA-His plasmid to achieve overexpression and purification of His-tagged CaeA, primers

CaeAHisF, which contained a sequence to add an in-frame N-terminal His-10 tag to the encoded CaeA protein, and CaeAHisR were used to amplify *caeA* from the genomic DNA template of *B. longum* subsp. *longum* NCIMB 8809 using Taq PCR master mix. The generated amplicon was digested with EcoRV and XbaI, and ligated to pNZ8150 digested with ScaI and XbaI. The ligation mixture was introduced into *L. lactis* NZ9000 by electroporation with Cm^R selection and positive clones were confirmed by colony PCR using Extensor PCR Master Mix and recombinant plasmid integrity was confirmed by DNA sequencing. For overexpression, 400 ml of M17 broth supplemented with 0.5 % glucose was inoculated (2 % v/v) with *L. lactis* NZ9000-pNZcaeA-His and incubated at 30°C until an OD_{600nm} of 0.5 was reached. Protein production was induced with purified nisin (5 ng ml^{-1}) for 2 hours. Cells were then harvested by centrifugation and the His-tagged CaeA protein was purified using the PrepEase His-tag protein purification kit (USB, Germany). Protein eluate fractions were analysed by SDS-polyacrylamide gel electrophoresis on a 12.5 % polyacrylamide gel (54) with the Color Prestained Protein Standard, Broad Range (11-245 kDa) ladder (New England BioLabs, USA). Polyacrylamide gels were then fixed and stained using a Coomassie Brilliant Blue to indicate which fractions contained the purified protein. Protein aliquots were dialysed overnight in 50 mM NaH_2PO_4-K_2HPO_4 buffer pH 7 using dialysis tubing (Medicell Membranes Ltd., London, United Kingdom) to remove imidazole remaining from the protein purification. The amount of protein in each aliquot was determined by the Bradford Assay (Sigma–Aldrich) after dialysis (55).

HPLC reactions. For High Performance Liquid Chromatography (HPLC) reactions, potential substrates methyl ferulate, ethyl ferulate, methyl *p*–coumaric acid, methyl sinapinate and methyl caffeic acid were dissolved in 96 % ethanol to generate 20 mM stock solutions. Reactions were carried out in 20 mM morpholinepropanesulfonic acid (MOPS) pH 7.5 with the substrates present at a 1 mM final concentration and 15 µg of CaeA protein per reaction in a final reaction volume of 1 ml. Potential substrates were also incubated in buffer without CaeA as a negative control. All reactions and negative controls were incubated at 37°C for 16 hours and were terminated by the addition of 370 µl ethyl acetate (Fisher Scientific) followed by centrifugation at 12,000 x *g*. The upper phase was then removed to a new tube and a further 370 µl of ethyl acetate was added,

followed by mixing and centrifugation at 12,000 x *g*. This second extraction was then used for analysis. The cinnamic acids and esters were detected, separated on an Agilent 1200 series LC instrument coupled with an MSD Trap XCT Ultra Ion Trap mass spectrometer. Mobile phase A consisted of water + 0.1 % formic acid and mobile phase B consisted of Acetonitrile + 0.1 % formic acid. A highly refined and optimised gradient method was developed to separate all of the cinnamic acids and esters, and this was achieved in a 47 min run. The chromatography column used for separation was an Agilent Eclipse XDB C-18 column (150 mm x 4.6 mm), and the column oven was maintained at 40°C. An injection volume of 5 µL was used for all injections with ethanol used as a needle wash and UV detection was completed in parallel to mass spectrometry as a detection system. UV wavelengths of 280 nm and 320 nm were selected for measurement purposes.

For mass spectrometry-based detection, positive alternating mode was used, acquiring data in both positive and negative mode, though in general the detected analytes were more suited to negative mode analysis. A scan range of 100 – 2200 m/z was used with a capillary voltage of -3500 V, Nebuliser pressure of 50 psi, Dry gas (Nitrogen) was utilized at 10 L/minute, a drying temperature of 350°C was used and an m/z value of 220 was employed as the set target mass. The skimmer was set to 40 V, while the capillary exit was at 107.5 V.

Substrate specificity assay. Enzyme reactions were carried out at 37°C in 0.1 M NaH_2PO_4- K_2HPO_4 buffer containing 0.6 % (v/v) Triton–X100 and 1.1 mg/ml of gum arabic (Sigma – Aldrich) at pH 7.5. 20 mM stock solutions of *p*-Np acetate, *p*-Np butyrate, *p*-Np octanoate and *p*-Np dodecanoate were prepared in 1:4 (v/v) acetonitrile: isopropanol. All reactions had a final substrate concentration of 6 or 12 µg/ml CaeA protein in a final reaction volume of 1 ml. Esterase enzymatic activity was measured by the release of *p*-Np from the substrates at the pH-independent wavelength 348nm. Reactions were terminated after 30 s by the addition of 25 µl of concentrated HCl (36 %) (Sigma–Aldrich). The rate of enzyme activity was calculated as µmol min^{-1} mg^{-1} of *p*-Np released. The maximal enzyme activity observed was then defined as 100 % and the relative activity for each reaction was calculated accordingly.

Hydrolysis of ethyl ferulate, methyl ferulate, methyl *p*-coumaric, methyl sinapinate or methyl caffeate was determined using *p*-Np as a proton sink as previously described with modifications (56). A 10 mM stock solution of each substrate dissolved in 96 % ethanol was prepared. A 10 mM stock solution of *p*-Np (Sigma-Aldrich) was used to prepare 1 mM NaH_2PO_4/ K_2HPO_4 buffer (pH 7) with *p*-Np at a final concentration of 0.44 mM. Assays were carried out in this buffer with 6 or 12 µg/ml CaeA and substrates at a final concentration of 1 mM in 200 µl at 37°C for 2 hr. The rate of the enzyme activity was calculated as µmol min^{-1} mg^{-1} of HCA released with standard curves for each corresponding HCA.

Optimal Temperature, pH and ions assay. A 20 mM stock solution of *p*-Np butyrate substrate was prepared in 1:4 (v/v) acetonitrile: isopropanol and 0.3 % (v/v) Triton–X100 (All from Sigma-Aldrich). Enzymatic assays were performed at 20°C, 25°C, 30°C, 37°C, 40°C, 50°C, 55°C for 30 s in 0.1 M NaH_2PO_4- K_2HPO_4 buffer at pH 7.5 with 6 µg/ml CaeA protein and a final concentration *p*-Np butyrate of 2 mM (100 µl) in a total reaction volume of 1 ml.

For optimum pH assays, a stock of 20 mM *p*-Np butyrate was prepared in 1:4 (v/v) acetonitrile: isopropanol. Impact of pH on enzyme activity was determined at 37°C in 0.2 M citric acid phosphate buffer (pH 3 – 5), 0.1 M NaH_2PO_4/ K_2HPO_4 buffer (pH 5 – 8) and 50 mM Tris HCL (7 – 9). All buffers also contained 0.6 % (v/v) Triton–X100 and 1.1 mg/ml gum arabic. The pH-variable assays were performed for 30 s with 6 or 12 µg/ml of protein with a final concentration of *p*-Np butyrate of 2 mM in a total reaction volume of 1ml. For both assays, rate of enzyme activity was calculated as µmol min^{-1} mg^{-1} of *p*-Np released. The maximal enzyme activity was then defined as 100 % and relative activity for each reaction was calculated. Enzymatic activity was measured at the pH independent wavelength 348nms.

The effect of metal ions on enzyme activity was also tested. Enzyme reactions were carried out at 37°C in 0.1 M NaH_2PO_4/ K_2HPO_4 buffer pH 7.5 in a microtiter plate. A stock of 20 mM of each ion was prepared in water. A stock of 20 mM *p*-Np butyrate was prepared in 1:4 (v/v) acetonitrile:isopropanol. Assays were performed in a final volume of 200 µl for 10 min with a final concentration of 6 µg/ml of protein and 2mM *p*–Np

butyrate. Ions were at a final concentration of 1 mM. Enzymatic activity was measured in all assays by the release of *p*-Np at the pH-independent wavelength of 348nm after 10 min. The rate of the enzyme activity was calculated as µmol min^{-1} mg^{-1} of *p*-Np released. The maximal enzyme activity was then defined as 100 % and relative activity for each reaction was then calculated.

HPAEC-PAD analysis. The feruloyl glucose substrate was dissolved in ethanol. Reactions were carried out in 0.1 M sodium phosphate pH 7.5 with the substrate at a 0.5 mg/ml final concentration and 15 µg of CaeA in a final reaction volume of 1 ml. A negative control including just feruloyl glucose and buffer (i.e. without enzyme) was also performed. Reactions and negative controls were incubated at 37°C for 16 hours and terminated by heating the sample at 98°C for 2 min. Standard solutions of 1 mg/ml glucose prepared in water and 0.5 mg/ml feruloyl glucose in ethanol were used. Standards and reactions were freshly prepared immediately prior to analysis. Samples were stored at 4°C before their assessment by High-Performance Anion Exchange Chromatography – Pulsed Amperometric Detection (HPAEC-PAD) analysis, which was performed employing a Dionex ICS-3000 system (Sunnyvale, CA) as follows. A 25 µl aliquot of each of the esterase reactions was separated on a CarboPac PA1 analytical exchange column (250 mm x 4 mm) with a CarboPAC PA1 guard column (50 mm x 4 mm) and a pulsed electrochemical detector (ED40) in the PAD mode. All columns and detectors were acquired from Dionex. Elution was carried out at a constant flow-rate of 1.0 ml min^{-1} at 30°C using the following eluents: eluent A, 200 mM NaOH; eluent B, 100 mM NaOH with 550 mM Na acetate and eluent C, MilliQ water. Analysis was performed using a linear gradient of sodium acetate with 100 mM NaOH from 0 min to 50 min, 0 mM; from 50 to 51 min, 100 mM; from 56 to 61 min.

2.4. Results

Identification of an esterase-encoding gene conserved among members of the *B. longum* subsp. *longum* taxon. *B. longum* subsp. *longum* has the capacity to metabolise plant carbohydrates; for example, arabinofuranosidases have been annotated and/or studied from strains in this taxon (12, 57, 58). However, no esterases, enzymes involved with the removal of HCAs from plant phenolics, have curently been studied from this taxon. An *in silico* search for an esterase gene in the available genome sequences of members of the *B. longum* subsp. *longum* taxon using Blastn revealed a highly conserved locus (B8809_1751 – B8809_1762 in *B. longum* subsp. *longum* NCIMB8809), predicted to be involved in plant-derived oligosaccharide degradation within the *B. longum* subsp. *longum* taxon (12, 21, 59) **(Figure 2.1)**. The locus includes genes predicted to encode (i) five arabinofuranosidases (B8809_1754, B8809_1757 – BB8809_1760), enzymes that are known to release arabinose moieties from certain plant polysaccharides such as arabinoxylan and arabinan; (ii) four ABC transporter permeases and a solute binding protein, which are presumed to be involved in the transport of arabinose into the cell (BB8809_1751 – 1753, BB8809_1761-1762); (iii) an esterase (BB8809_1755), and (iv) a LacI-type regulatory protein (B8809_1756), which may be responsible for transcriptional control of the genes of this locus. The gene product of B8809_1754, or AbfII2 as previously designated, exhibits 51% similarity to a previously characterised arabinofuranosidase from *Streptomyces avermitilis* NBRC14893 (60). The annotated esterase (corresponding to locus tag BB8809_1755) from *B. longum* subsp. *longum* NCIMB 8809 was selected for analysis and designated *caeA* (for cinnamoyl acid esterase A, its function as will be outlined below). HHPred-based analysis predicts that the CaeA protein shares a conserved structure with esterases from several bacterial species, while BlastP searches indicated that CaeA contains a conserved alpha-beta hydrolase domain which is typical of esterases (61).

Sequence alignment of CaeA with several experimentally validated esterases, including an esterase from *Bifidobacterium animalis* subsp. *lactis* with activity against chlorogenic acid (30), showed the presence of the conserved Gly–X–Ser–X–Gly esterase hydrolytic motif around the Ser-His-Asp catalytic triad. The active site Ser is at the center of the Gly-X-Ser-X-Gly motif **(Supplementary figure S2.1)**. However, these esterases

exhibit low sequence similarity to CaeA, ranging from 27 to 33 %. CaeA is predicted to represent a cytoplasmic protein as based on SignalP prediction (62). Since the *caeA* gene is located within a genetic locus presumed to be involved in arabinoxylan and arabinan metabolism, we speculate that CaeA may be involved in the removal HCAs from the arabinose residues in arabinoxylan, arabinan and perhaps other plant carbohydrates. For this reason we wanted to confirm the suspected esterase activity of CaeA against model HCA substrates.

Heterologous expression and hydrolytic activity of CaeA. In order to assess if CaeA is able to hydrolyse ethyl ferulate, a model substrate for esterase activity (53, 63), *caeA* was cloned into the expression vector pNZ44 (64), to generate pNZ44caeA, and introduced into *Bifidobacterium breve* UCC2003 which does not contain a *caeA* homolog. *B. breve* UCC2003 WT, *B. breve* UCC2003 pNZ44 (negative control) and *B. breve* UCC2003 pNZ44caeA were then spot plated on to RCA supplemented with 0.1 % (vol/vol) ethyl ferulate and a zone of clearance was observed arround the spotted colonies, indicating the breakdown of ethyl ferulate in the case of *B. breve* UCC2003 pNZ44caeA, indicating expression of esterase activity supplied by the CaeA protein, yet not for *B. breve* UCC2003 WT or *B. breve* UCC2003 pNZ44 **(Figure 2.2)**. This result therefore supports the notion that CaeA is a functional esterase capable of hydrolysing ethyl ferulate.

Protein purification of CaeA and enzymatic activity against model HCA substrates. In order to assess the enzymatic activity and substrate specificity of CaeA, a His-tagged version of this protein was expressed in *L. lactis* NZ9000 and purified by Ni–affinity chromatography. This His-tagged CaeA protein was shown to exhibit an approximate size of 36 kDa when analysed by SDS-PAGE **(Figure 2.3)**, in agreement with the molecular mass (35.57 kDa) of the protein including the N-terminal His_{10}-tag as determined by the ExPASY molecular weight calculator (65). An additional band, presumed to be a co-eluted protein, is observed in the gel just above the CaeA protein band. For this reason we used a negative control in all enzyme assays described below, represented by a nisin-induced *L. lactis* NZ9000 culture carrying the empty expression vector. The purified His-tagged CaeA protein was tested for esterase activity against several substrates (i.e. methyl

ferulate, ethyl ferulate, methyl caffeate, methyl *p*–coumaric acid and methyl sinapinate) to determine substrate specificity, and to assign CaeA to either of the esterase sub-groups A, B, C or D. CaeA was shown to release the associated HCA from methyl ferulate, ethyl ferulate, methyl *p*–coumaric and methyl caffeate, while no noticeable activity was found against methyl sinapinate **(Table 2.3 & Supplementary Figure S2.2)**. These results indicate that CaeA can be classified as a type B feruloyl esterase (42). A subsequent assay was employed to quantify the amount of HCA released once the ester bond of the HCA esters is hydrolysed. The obtained results demonstrate that CaeA can release HCA from methyl ferulate, ethyl ferulate and methyl caffeate, while there was no detectable activity against methyl sinapinate. Methyl *p*-coumaric and chlororgenic acid were also tested, however; due to the intrinsic properties of these substrates HCA release could not be accuratley measured in this assay. CaeA was most active towards methyl ferulate under these conditions **(Figure 2.4)**. This contrasts with the activity of the esterase from *Lactobacillus plantarum* WCFS1, which was shown to exhibit more activity towards methyl caffeate (31). CaeA was able to cleave methyl caffeate, yet was less efficient with a relative activity of 36 % as compared to 68 % activity towards ethyl ferulate. CaeA was furthermore shown to cleave the ester bond of 6-O-feruloyl glucose, thereby releasing glucose as detected by HPAEC–PAD **(Figure 2.5)**.

Esterase versus lipase substrate range of CaeA. The hydrolytic activity of CaeA towards several colorimetric substrates containing 4 to 12 carbons was also determined. 'True' esterases generally recognize substrates that contain less than six carbons, whereas lipases may be active on substrates containing more than six carbons (39). The activity in these colorimetric assays was determined by the amount of released *p*-Np using a photospectrometer at 348nm. The mean specific activity of CaeA on *p*-Np butyrate is 8.35 µmol min^{-1} mg^{-1}. The activity of CaeA towards *p*-Np acetate, *p*-Np octanoate and *p*-Np dodecanoate was then determined relative to that observed for *p*-Np butyrate (which was set at 100 %) **(Figure 2.6)**. From the obtained results it is clear that CaeA has a substrate preference for *p*-Np butyrate and appears to be functioning as a 'true' esterase since the enzyme elicits substantially reduced activity towards the longer chain substrates with just 13.7 % and 15.7 % activity against *p*-Np octanoate (8 carbons in length) and *p*-Np-

dodecanoate (12 carbons in length), respectively. CaeA also exhibits a lower relative activity of 40.8 % towards *p*-Np acetate. In contrast, other esterases from several lactobacilli species and *B.* animalis subsp. *lactis* DSM 10140 have been shown to exert maximal hydrolytic activity towards the shorter *p*-Np acetate, though exhibit low activity towards *p*-Np octanoate, a property they have in common with CaeA (66). Nonetheless, CaeA is not unique in exhibiting its preferred actitivy towards *p*-Np butyrate (31, 67).

Optimum pH, Temperature and effect of ions on CaeA. The biochemical properties of CaeA were investigated to ascertain the reaction conditions for optimal activity of CaeA. The optimum temperature and pH were determined by measuring the release of *p*-Np, a colourimetric substrate at 348nm, from *p*-Np butyrate. Relative activity for each condition was calculated by normalising the data to the highest specific activity of CaeA, 12.65 μmol min^{-1} mg^{-1} for pH and 25.40 μmol min^{-1} mg^{-1} for temperature, and expressing the data as a percentage relative to this value. The optimal temperature for CaeA was found to be 40°C and the optimum pH was 7.5 **(Figure 2.6)**. The lowest activity of CaeA was observed at 55°C and pH 4.5, conditions that diminished activity to 25 % and 11%, respectively. Nontheless, CaeA appears to be a versatile enzyme, exhibiting activity across a rather wide range of temperatures and pH conditions. The effect of ions and detergents on CaeA was also investigated **(Figure 2.7)**. No substantial impact on esterase activity was noted except for the addition of Cu^{2+} which reduced activity to 7%. Reduction of esterase activity by Cu^{2+} has been reported elsewhere in the literature (33, 68, 69).

2.5. Discussion

Members of the *B. longum* subsp. *longum* taxon have been specifically associated with complex plant carbohydrate metabolism, making these plant-derived glycans candidate prebiotics for these bifidobacteria. HCAs are frequently found esterified to plant carbohydrates that are indigestible to the human host and are therefore more likely released in the colon by particular members of the gut microbiota (40, 70-73). Much work on plant-derived poly/oligosaccharide metabolism in bifidobacteria has focussed on arabinofuranosidase and xylanase, β-glucosidase activities (9, 57, 74-76). However, since

HCAs are a component of plant carbohydrates it is also important to investigate if bifidobacterial produce esterases.

In the current study we identified and biochemically characterised a novel HCA esterase from *B. longum* subsp. *longum* NCIMB 8809. Significantly, this esterase-encoding gene was located within a highly conserved locus within the genome of all analyzed members of this taxon. The *B. longum* subsp. *longum* taxon is known to metabolise plant oligosaccharides such as arabinoxylan and arabinan (15), and therefore the genetic location of this esterase in an arabinoxylan/arabinan metabolism cluster suggests that HCAs that are attached to the arabinose residues of plant carbohydrates are cleaved off as part of the process of metabolising these complex plant cell wall carbohydrates (12, 21). This co-location of an esterase-encoding gene within a polysaccharide utilisation locus is similarly reported for other species of bacteria in the gut microbiota such as *Bacteroides* species (77, 78). Furthermore, these plant cell wall glycans have been reported to act as prebiotics stimulating bifidobacterial growth in the gut (79-81). In order to allow enzymatic access to these dietary polysaccharides bifidobacteria are likely to require an esterase to remove HCAs prior to the metabolism of the carbohydrate moiety. Nonetheless, Riviere and colleges found that the presence or absence of an esterase gene in bifidobacterial strains did not correlate to a strain's ability to metabolise arabinoxylo-oligosaccharides (15). It must be noted that the actual specific constituents of arabinoxylan and AXOS are highly variable (21, 82), and that an esterase may therefore not be needed by all strains to metabolise certain AXOS constituents.

We demonstrated that the purified CaeA esterase was active against a number of different substrates, such as feruloyl glucose and *p*-Np butyrate. Heterologous expression of CaeA in *B. breve* UCC2003 also conferred esterase activity to a bifidobacterial strain normally devoid of esterase activity. CaeA contains the general characteristic esterase G-X-S-X-G motif, Ser-Asp-His catalytic triad and the conserved alpha/beta hydrolase structure typical of esterase and lipases. CaeA is a 'true' esterase rather than a lipase as it elicits a preference for smaller carbon backbone substrates less than six carbons. It has previously been reported that bifidobacterial esterases from *B. animalis* subsp. *lactis* WC

0432 and *B. animalis* subsp. *lactis* DSM 10140 exhibit hydrolytic activity against chlorogenic acid and artificial HCA-containing substrates (30, 66).

Certain bifidobacterial taxa may be able to release HCAs from plant oligosaccharides in the gut and may make these phenolic compounds available for their own metabolic use, to the human host and/or to other members of the gut microbiota. HCAs have been reported to act as external electron acceptors and may thus provide an energetic advantage for heterofermentative lactobacilli by increasing the amount of ATP and NADH regeneration (36, 37). Increased bioavailability of the HCAs may also have consequences and/or reflect the disease state of the host. In diabetes-resistant rat models lactobacilli and bifidobacteria were found to be more abundant compared to diabetes-sensitive rats (83); lactobacilli with an increased capability of HCA hydrolysis were isolated from the same patient sample set (26). However, it should be noted that conclusive proof for HCA metabolism by bifidobacteria is as yet lacking.

Similar to the esterase from *B. animalis* subsp. *lactis* WC0432, CaeA is presumed to be an intracellular enzyme as based on the lack of an obvious protein secretion signal (30). Therefore, whether certain bifidobacteria increase bioavailablity of HCAs to the host still remains unclear. A limitation of our study is that we did not employ plant oligosaccharide substrates substituted by HCAs to test this as the plant oligosaccharide isolation process usually removes HCAs. Future work should determine if bifidobacteria can metabolise HCAs, and if so, assess the consequences of this ability for bifidobacterial physiology in the gut environment. Furthermore, the question should be addressed as to whether or not bifidobacteria release HCAs in their environment to make them available to the host or other gut microbes.

In conclusion, this study has found that members of the *B. longum* subsp. *longum* taxon possess a highly conserved esterase-encoding gene, which is co-located with genes associated with plant poly/oligosaccharide degrading enzymes on the *B. longum* subsp. *longum* genome. Therefore, CaeA is likely an important enzyme in the metabolism of plant oligosaccharides by *B. longum* subsp. *longum* taxon. CaeA is a true esterase capable of cleaving several HCA and esterase model substrates and thus bifidobacteria a likely can release HCAs from plant oligosaccharides. *B. longum* subsp. *longum* is the second

known bifidobacterial species able to express an esterase that may remove HCAs from plant carbohydrates.

2.6. Author Contributions

SMK designed experiments, carried out experiments, analysed experimental data, wrote the manuscript. JOC designed experiments, carried out experiments, analysed experimental data. MK designed experiments, carried out experiments and analysed experimental data. DVS designed experiments and wrote the manuscript.

2.7. Acknowledgements

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2.8. Tables and figures

Table 2.1. Bacterial strains and plasmids used in this study

Bacterial strain/ plasmid	Features	Reference
Lactococcus lactis		
NZ9000	MG1363 *pepN::nisRK;* nisin inducible overexpression host	(de Ruyter et al., 1996)
NZ9000-pNZ8150	NZ9000 containing plasmid pNZ8150	(Mierau and Kleerebezem, 2005)
NZ9000-pNZcaeA-His	NZ9000 containing pNZ8150 expressing *caeA*	This study
Bifidobacterium longum* subsp. *longum		
NCIMB 8809	Nursling stool isolate	NCIMB, Aberdeen, Scotland
Bifidobacterium breve		
UCC2003	Nursling stool isolate.	(Maze et al., 2007)
UCC2003-pNZ44caeA	UCC2003 containing pNZ44caeA	This study
Plasmids		
pNZ8150	Cm^R, translational fusion vector induced by nisin.	(Mierau and Kleerebezem, 2005)
pNZcaeA-His	*caeA* with a His tag cloned downstream of the nisin inducible promoter on pNZ8150.	This study
pNZ44	Cm^R, derivative of pNZ8048 with a constitutive promoter.	(McGrath et al., 2001)
pNZ44caeA	Cm^R, pNZ44 derivative containing *caeA*	This study

Table 2.2. Oligonucleotide sequences used in this study

Function	Primer	Sequence
Cloning of *caeA* into pNZ44	CaeAF	gctcga**ccatgg**Atcagcgttcatcattcg*
Cloning of *caeA* into pNZ44	CaeAR	ctctgc**tctaga**gaatgtccgcgcagccgtac
Cloning of *caeA* with His tag into pNZ8150	CaeAHisF	cctgca**gatatc**atgcatcaccatcaccatcaccatcaccatcacgac atcaaaccgtgggaatac
Cloning of *caeA* with His tag into pNZ8150	CaeAHisR	ctctgc**tctaga**gaatgtccgcgcagccgtac

*Restriction enzyme sites are highlighted in bold.

Table 2.3. HPLC anaylsis of CaeA activity against HCA substrates

HCA substrate	Activity
Methyl ferulate	+
Ethyl ferulate	+
Methyl *p*-coumaric acid	+
Methyl caffeate	+
Methyl sinapinate**	-

**No hydrolysis evident in the case of Methyl Sinapinate.

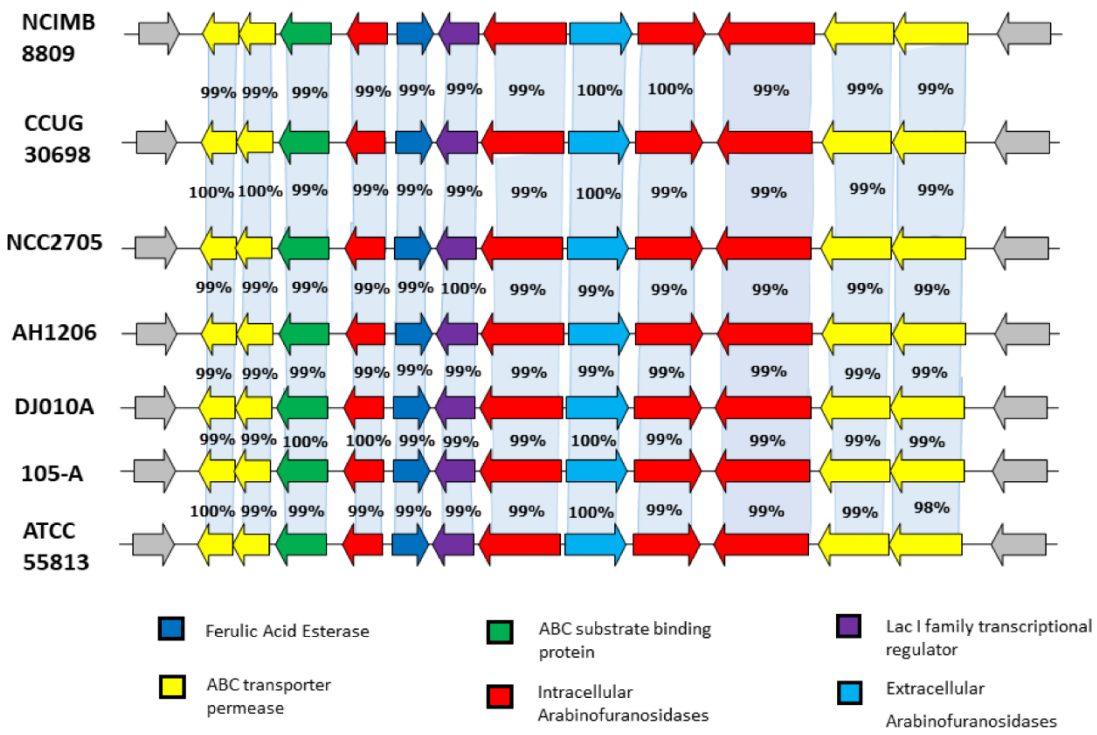
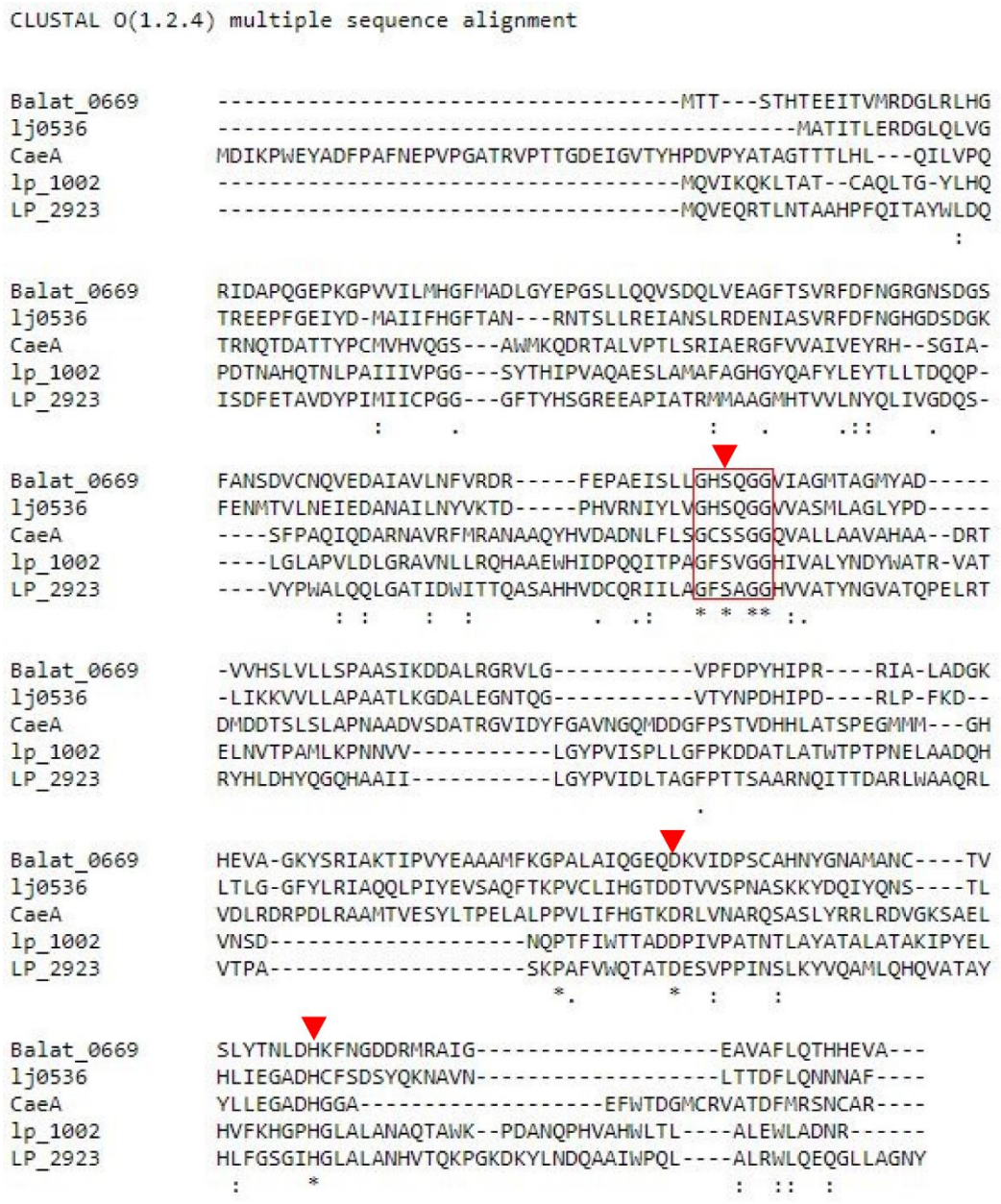


Figure 2.1. Comparison of the conserved plant oligosaccharide degradation locus amongst complete and available genomes of the *B. longum* subsp. *longum* taxon.

B. longum subsp. *longum* strains are indicated in bold. The arrows represent open reading frames which are proportional to open reading frame length. The nucleotide identity of each of the open reading frames is calculated as a percentage of identity to the equivalent open reading frame in *B. longum* subsp. *longum* NCIMB 8809.



Supplementary Figure S2.1. Esterase multiple sequence alignments.

Multiple sequence alignment of CaeA (B8809_1755) from *B. longum* subsp. *longum* NCIMB 8809, Balat_0669 from *B. lactis* subsp. *animalis*, Lp_1002 from *Lactobacillus plantarum* WCFS1, Lp_2923 from *L. plantarum* WCFS1 and lj0536 from *Lactobacillus johnsonii* N6.2. The (Gly – X – Ser – X – Gly) esterase motif is highlighted in the red box and the Ser-Asp – His triad residues are indicated by the red triangles.

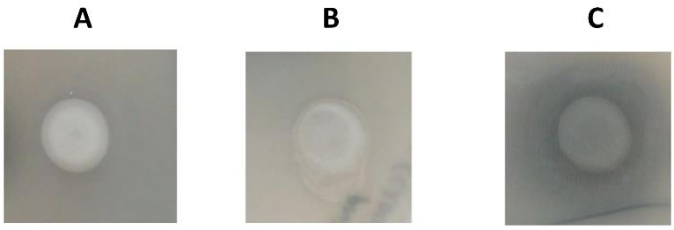


Figure 2.2. Esterase Activity Plate Assay.

Growth of *B. breve* UCC2003 (A), *B. breve* UCC2003 pNZ44 (B) and *B. breve* UCC2003 pNZ44_CaeA (C) on RCA supplemented with 0.1 % (w/v) ethyl ferulate acid. A zone of clearing indicates esterase activity.

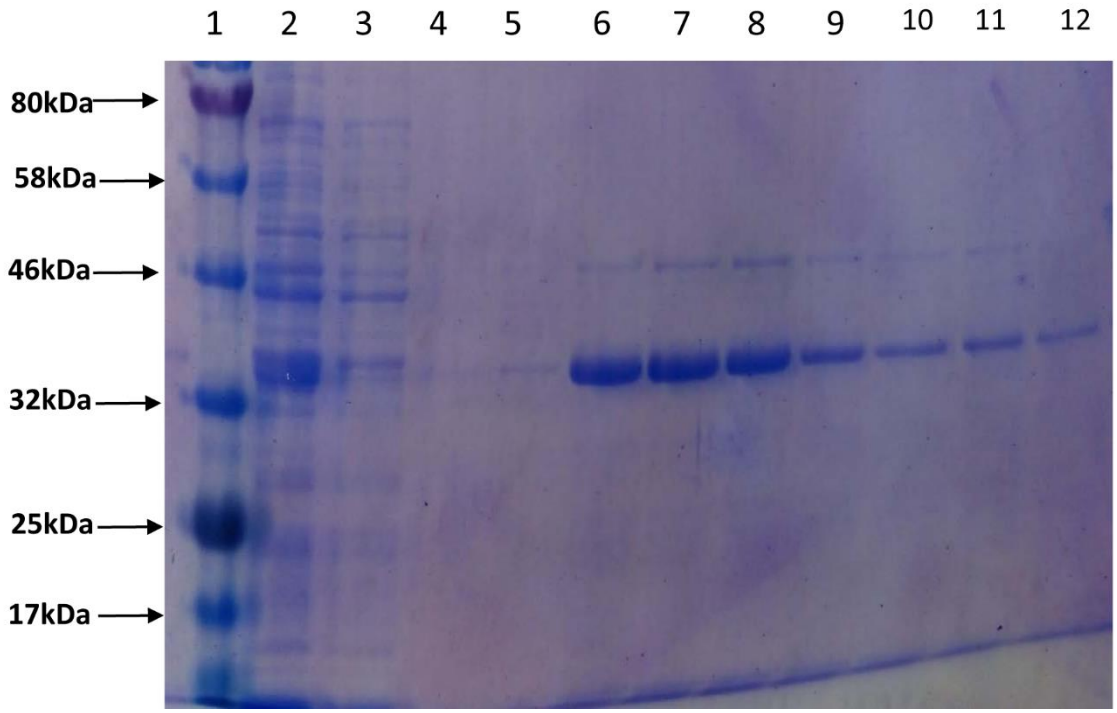
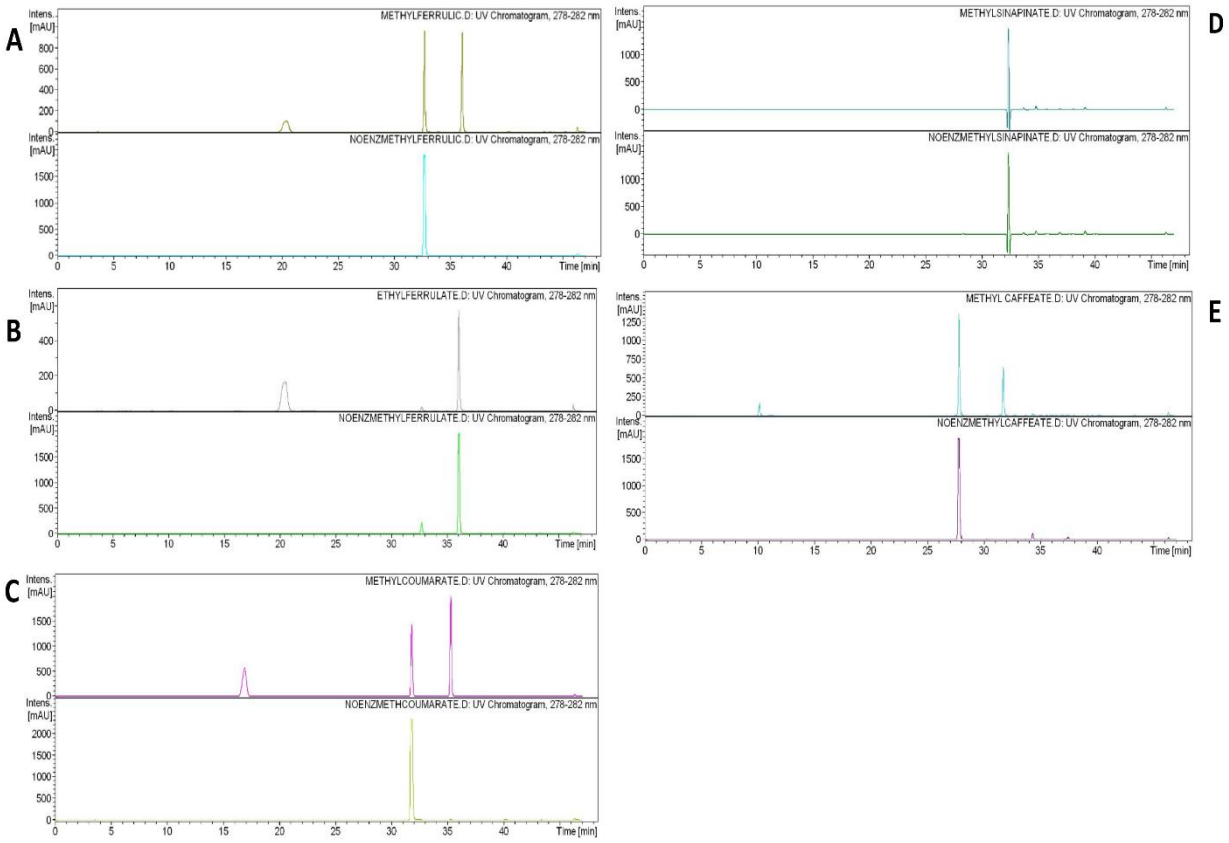


Figure 2.3. Purification of CaeA.

The 12.5 % SDS-PAGE gel including protein standard ladder (lane 1), supernatant (lane 2), column wash (lane 3), column wash (lane 4) and elution aliquots (lanes 5 – 12).



Supplementary Figure S2.2. HPLC analysis of the activity of CaeA against hydroxycinnaminic acid substrates.

Enzyme reactions were carried out in 20 mM morpholinepropanesulfonic acid (MOPS) pH 7.5 with the substrates present at a 1 mM final concentration and 15 µg of CaeA protein per reaction in a final reaction volume of 1 ml. All substrates were also incubated in buffer without CaeA as a negative control. All reactions and negative controls were incubated at 37°C for 16 hours and were terminated by the addition of 370 µl ethyl acetate. Assays were performed in duplicate. CaeA activity against methyl ferulate and corresponding no enzyme control **(A)**, ethyl ferulate and no enzyme control **(B)**, methyl *p*-coumaric acid and no enzyme control **(C)**, methyl sinapinate and no enzyme control **(D)**, and methyl caffeic acid and no enzyme control **(E)**.

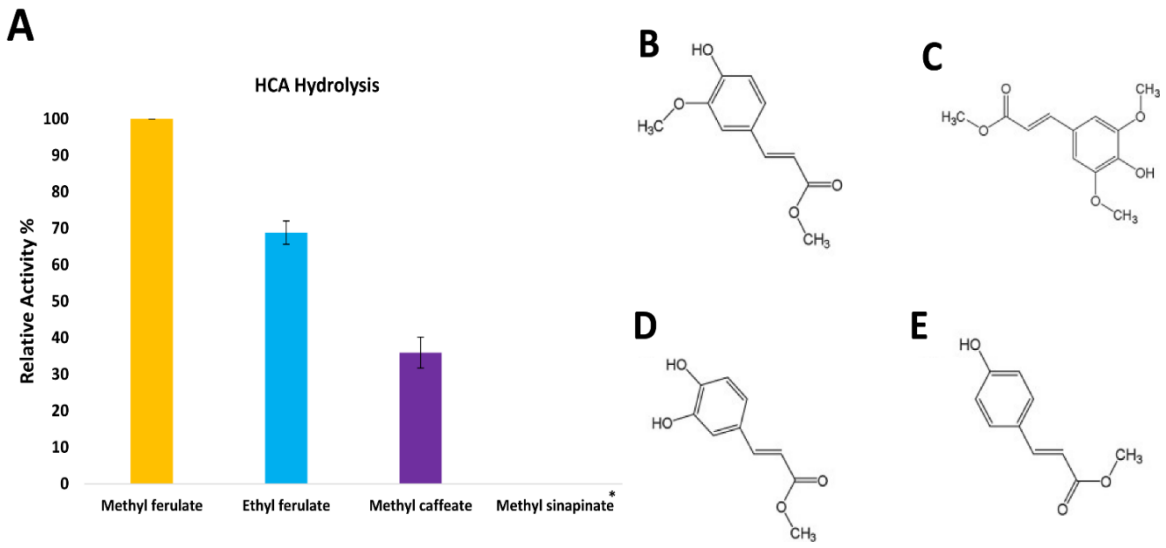


Figure 2.4. Release of HCAs from methyl ester substrates.

The relative activity of CaeA against synthetic HCA esters; methyl ferulate, ethyl ferulate and methyl caffeate **(A)**. Assays were performed in 1mM NaH_2PO_4 K_2HPO_4 pH 7 at 37°C for 2 Hr with a protein concentration of 6µg/ml. Data is representative of mean values and standard error of the mean. * No measurable enzyme activity was found against methyl sinapinate. The structure of Methyl ferulate **(B)**, Methyl sinapinate **(C)**, Methyl Caffeate **(D)** and Methyl *p*-Coumarate **(E)**. These structures were partially adapted from a previous publication (63).

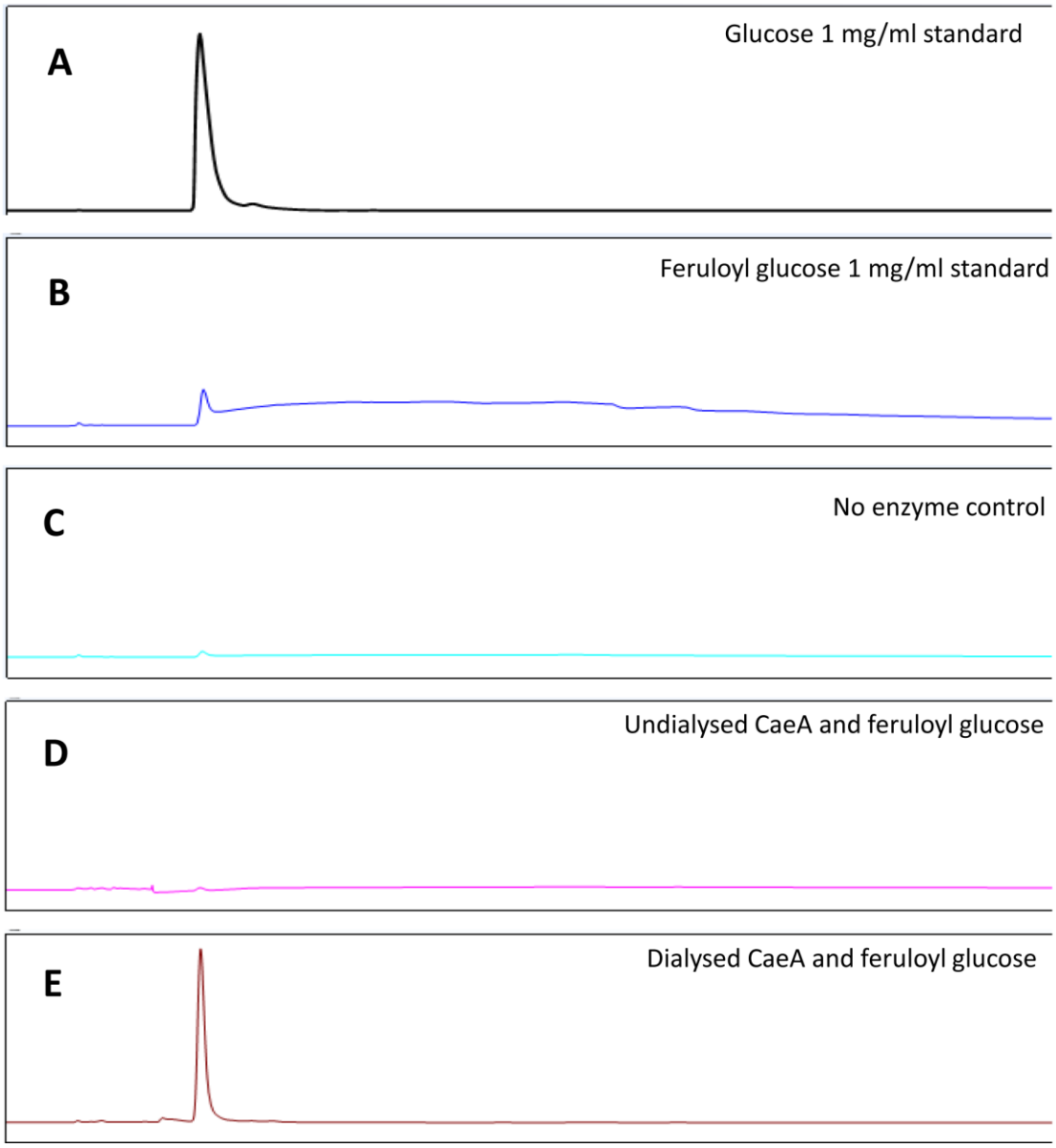


Figure 2.5. HPAEC – PAD analysis of CaeA activity against feruloyl glucose.

Glucose standard (1 mg/ml) **(A)**. Feruloyl glucose (0.5mg /ml) standard **(B)**. No enzyme control where feruloyl glucose is incubated for 16 Hrs at 37 °C **(C)**. Undialysed CaeA incubated with feruloyl glucose after 16Hrs at 37°C **(D)**. Dialysed CaeA incubated with feruloyl glucose after 16Hrs at 37°C **(E)**. Assays were carried out in 0.1M sodium phosphate buffer at pH 7.5 with 15 µg/ml of protein.

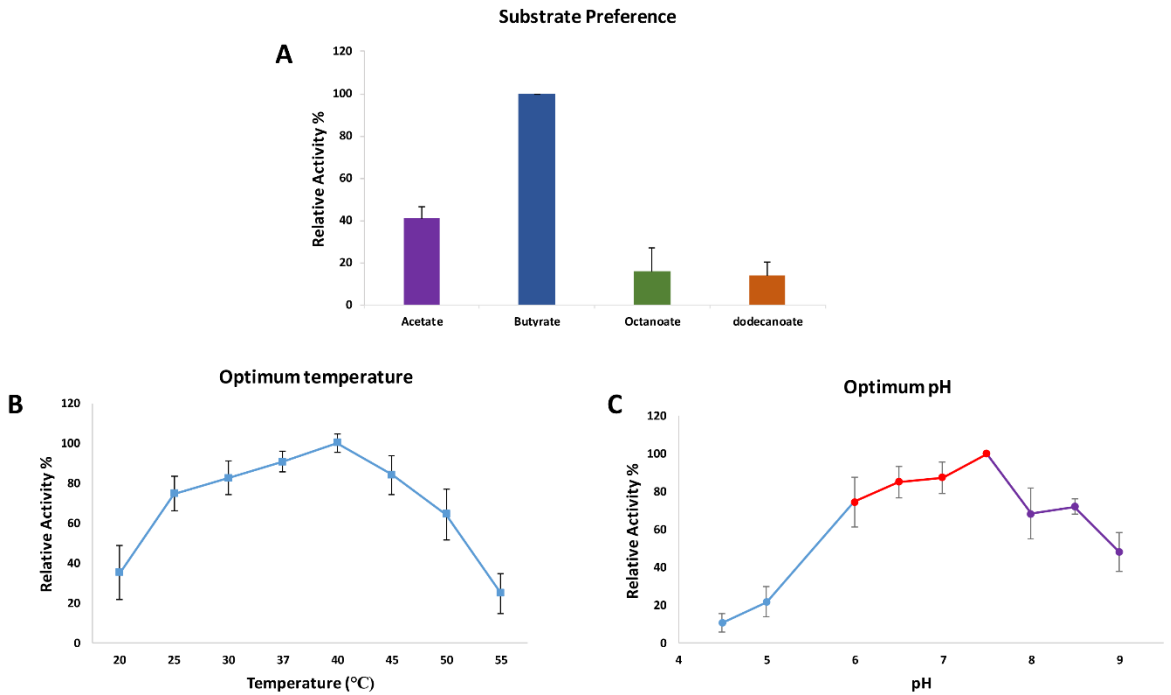


Figure 2.6. Determination of substrate specificity, pH optimum and temperature optimum of CaeA.

Maximal observed activity on *p*-Np buytrate was defined as 100 %. Optimum substrate preference assays were carried out in 0.1 M NaH_2PO_4 K_2HPO_4 buffer pH 7.5 at 37 °C **(A)**. Optimum temperature assays were performed in 0.1 M NaH_2PO_4 K_2HPO_4 buffer pH 7.5 at various temperatures to ascertain the optimum temperature for CaeA **(B)**. Optimum pH assays were performed at 37 °C in 0.2 M Citric phosphate buffer (Blue), 0.1 M NaH_2PO_4 K_2HPO_4 buffer (Red) and 50 mM Tris-HCL buffer (Purple) **(C)**. All assays were carried out with *p*-Np butyrate as the substrate. Data is representative of mean values and standard error of the mean.

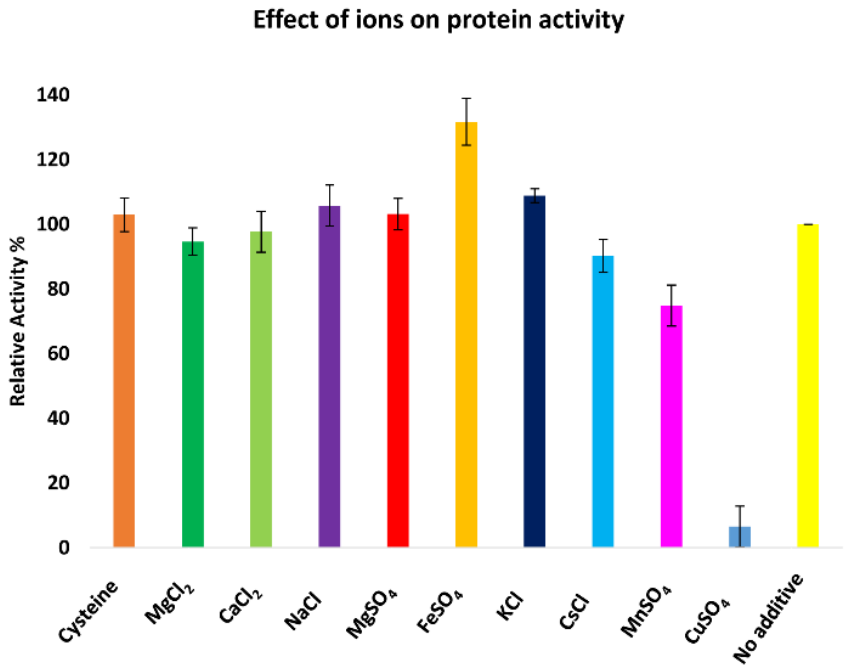


Figure 2.7. Investigation of the effect of ions on the activity of CaeA.

Investigation of the effect of ions on the activity of CaeA. Ions were added at a 1 mM final concentration and 100 % activity was defined as the activity of CaeA in the absence of any additive. Assays were performed at 37°C in 0.1 M NaH_2PO_4 K_2HPO_4 buffer pH 7.5 using *p*-Np butyrate as a substrate. Data is representative of mean values and standard deviation. Maximal activity was defined as 100 %.

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Chapter V

Bifidobacterial biofilm formation is a multifactorial adaptive phenomenon in response to bile exposure

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Dr. Francesca Bottacini was resposbile for RNAseq bioinformatics and analysis.

Dr. G.A. Lugli, A. Viappiani, Dr. F. Turroni and Prof. M Ventura carried out RNA extraction, cDNA synthesis, cDNA library preparation and RNAseq experimental design.

5.1. Abstract

In the current study, we show that biofilm formation by various strains and species belonging to *Bifidobacterium*, a genus that includes gut commensals with reported health-promoting activities, is induced by high concentrations of bile (0.5 % (w/v) or higher) and individual bile salts (20 mM or higher), rather than by acid or osmotic stress. The transcriptomic response of a bifidobacterial prototype *Bifidobacterium breve* UCC2003 to such high bile concentrations was investigated and a random transposon bank of *B. breve* UCC2003 was screened for mutants that affect biofilm formation in order to identify genes involved in this adaptive process. Eleven mutants affected in their ability to form a biofilm were identified, while biofilm formation capacity of an insertional mutation in *luxS* and an exopolysaccharide (EPS) negative *B. breve* UCC2003 was also studied. Reduced capacity to form biofilm also caused reduced viability when exposed to porcine bile. We propose that bifidobacterial biofilm formation is an adaptive response to high concentrations of bile in order to avoid bactericidal effects of high bile concentrations in the gastrointestinal environment. Biofilm formation appears to be a multi-factorial process involving EPS production, proteins and extracellular DNA release, representing a crucial strategy in response to bile stress in order to enhance fitness in the gut environment.

5.2. Introduction.

Biofilms are microbial communities that are attached to a surface and are enclosed/structured by an extracellular matrix (1). Biofilms may also form when free floating cells clump together or adhere to particulate matter, this being referred to as microcolony formation (2). Biofilm formation is a complex process involving multiple steps, including initial attachment, accumulation, maturation and dispersal (3). Initial attachment is reversible and can be driven by electrostatic interactions; attachment to a surface can also be mediated by cell wall-associated proteins that bind to a substrate-covered surface or extracellular DNA (eDNA) release, where DNA released by cell lysis coats the surface and changes surface properties to allow attachment (4, 5). The accumulation phase of a biofilm can be mediated by cell wall associated protein interactions or extracellular matrix (EM) secretion (5, 6). The EM of biofilms may be composed of extracellular polysaccharide (EPS), DNA and/or proteins (7-9). Finally, following maturation, individual cells may disperse from the biofilm to resume planktonic growth (10). Biofilm formation is often triggered in response to environmental stresses, such as nutrient starvation, antibiotics, pH and bile, or induced through quorum sensing systems, such as the Agr or autoinducer-2 (AI-2) systems (11-14).

Bifidobacteria are non-motile gut commensals, some of which are purported to exert health-promoting or probiotic properties (see review (15)). Particular bifidobacterial strains are for this reason included in certain 'functional foods' so as to bestow these benefits to the host that ingests them (16). However, whether bifidobacteria colonise from birth or are ingested as a probiotic they will encounter and must overcome stresses in the gastrointestinal tract (GIT), such as low pH, bile, osmotic stress and nutrient starvation, as well as compete with other members of the microbiota (17). Bile is present as a gradient along the GIT (40 mM to 0.5 mM), being highest in the small intestine and lowest in the colon (18, 19); however, bile/bile salt concentrations will vary greatly upon ingestion of (certain types of) food.

Bile and its constituent bile salts represent a major stress-inducing factor to bacteria in the GIT environment due to their bactericidal properties (19-21). There are different types of bile salts since primary bile salts such as chenodeoxycholic acid or

cholic acid can be conjugated with either a taurine or glycine before secretion. Primary bile salts can also be dehydroxylated by the gut microbiota to form secondary bile acids which can also be conjugated by taurine or glycine (20). Bile salts are bactericidal and target and disrupt the bacterial cell membrane (20). In bifidobacteria bile resistance mechanisms include efflux of bile salts by multi-drug transporters (22-24), compositional changes of the cell membrane (25-27), F_0F_1-ATPase proton efflux (28), changes in metabolism (29-31) and hydrolysis of bile salts (32, 33). Bile has previously been shown to induce biofilm formation in certain gut commensals, such as particular species/strains of *Bacteroides*, bifidobacteria and lactobacilli (34-36). Therefore, it is important to study biofilm formation in commensal strains, such as bifidobacteria, and to obtain insights into how they adjust to and survive bile stress, and how this contributes to gut colonisation.

Bifidobacteria have previously been shown to form microcolonies on the gut mucosal surface and food particulates isolated from the gut (37, 38). Currently, little is known about the molecular mechanisms of biofilm formation in bifidobacteria. Bile and bile salts at relatively high concentrations (0.5 % taurocholic acid and 5 % porcine bile) have previously been found to induce biofilm formation in bifidobacteria (34). In many bacterial species a specific quorum sensing signalling system is required for the induction of biofilm formation. For example, the AI-2 system involves LuxS, a S-ribosylhomocysteinase, producing AI-2, which is released extracellularly, and then sensed by the LuxP, LsrB or RbsB receptors of two component systems which in turn cause transcriptional induction of genes involved in eDNA release and polysaccharide production, among others, biofilm formation (39-41). Previously, AI-2 activity has been detected by several bifidobacterial species and strains, while in addition the over-expression of LuxS in *Bifidobacterium longum* subsp. *longum* NCC2705 has been linked to increased biofilm formation (42-44). The exposure and growth of *Bifidobacterium breve* UCC2003 to bile and bile salts has also been shown to cause increased transcription of *luxS* which is a homolog of the previously studied *luxS* in *B. longum* subsp. *longum* NCC2705 (24, 42). An insertion mutant in *luxS* in *B. breve* UCC2003 has previously been demonstrated to negatively affect gut colonisation ability in a mouse model (43). However, the effect of a *luxS* mutation on biofilm formation in *B. breve* UCC2003 was

not investigated. Besides these studies, essentially nothing is known about the molecular mechanisms of biofilm formation in bifidobacteria.

The aim of this study was to identify at what physiologically relevant concentrations of bile/bile salts biofilm formation is induced, and to identify genes involved in bifidobacterial biofilm formation. Our findings indicate that biofilm formation is a multi-factorial response to high concentrations of bile which is likely to be crucial for survival and colonisation of bifidobacteria within the gut environment.

5.3. Materials and Methods.

Bacterial strains, culture conditions, media.

All bacterial strains used in this study are listed in Table 4.1. Bifidobacterial strains were routinely cultured in reinforced clostridial medium pH 6.8 (RCM, Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) or reinforced clostridial agar (RCA, Oxoid Ltd.). RNAseq experiments were carried out using cultures that had been grown in filtered RCM (fRCM). All bifidobacterial strains were grown anaerobically in a modular atmosphere controlled system (Davidson and Hardy, Belfast, Ireland). Where required, media was supplemented with tetracycline (Tet, 10 µg ml^{-1}) or porcine bile, 0.5 % (w/v) or 1 % (w/v) (Sigma- Aldrich, Steinheim, Germany). Individual bile salts were purchased from Sigma-Aldrich.

Crystal Violet Biofilm Assay.

Overnight cultures of bifidobacteria (20 µl) were used to inoculate RCM supplemented with 0.5 % (w/v) or 1 % (w/v) porcine bile (Final volume 200 µl) in a 96 well microtiter plate. Biofilms were allowed to form for 24 Hrs at 37°C in anaerobic conditions and were then washed three times with deionised water to remove planktonic cells and left to dry for 1 hour. The biofilms were stained with 1 % crystal violet (100 µl) (Sigma-Aldrich) for 1 minute and then washed three times with deionised water to remove excess crystal violet

stain. Crystal violet stained biofilms were then solubilised with 5 % (v/v) acetic acid (100 µl) (Sigma-Aldrich) and the absorbance read at a wave length of 570 nm.

Screening of a mutant library for biofilm defective mutants.

A previously described transposon mutant library of *B. breve* UCC2003 (49, 50) was screened for mutants affected in their ability to from a biofilm. Individual transposon mutants were subcultured in RCM supplemented with tetracycline and RCM supplemented with 0.5 % and 1 % (w/v) porcine bile, to induce biofilm, for 24 Hrs. Biofilm formation was stained as described above. Transposon mutants that exhibited reduced biofilm formation were then selected for further analysis. The precise location of the transposon in a given mutant was then identified by iPCR as previously described (49, 50) or by arbitrary primed PCR as previously described with modifications (65, 66). Round one of arbitrary PCR was carried out with primers Arb 1, Arb 6 and either TnTetR1 or TnTetL1 (**Table 5.2**). The successful PCR reaction was then used in a second PCR reaction (round 2) using primers Arb2 and either TnTetR2 or TnTetL2 (**Table 5.2**). PCR reactions from iPCR reactions and round 2 arbitrary PCR reactions were then sequenced (Eurofins, Ebersburg, Germany) to identify transposon insertion with primers pMod-Fw-seq and pMod-Rv-seq.

DNA Manipulations.

DNA manipulations were carried out as previously described (67). Oligonucleotides used in this study were synthesised by Eurofins (Ebersberg, Germany) and are listed in **Table 5.2**. Genomic isolations from *B. breve* UCC2003 were performed as described previously (68). Inverse PCR and arbitrary PCR reactions to identify transposon insertion points, were carried out with the 2X Phusion Green HSII High Fidelity polymerase (Thermo-Scientific) and Q5 High Fidelity DNA polymerase (New England Biolabs), respectively. All PCR products were purified using the High Pure PCR Purification Kit (Roche). Restriction enzymes (Sigma Aldrich) and T4 DNA ligase (Promega) were used as stated in the manufacturer's instructions.

Transcriptomic analysis.

An overnight culture of *B. breve* UCC2003 in RCM was used to inoculate (1 % v/v) fRCM and this culture was grown until an $O.D._{600nm}$ between 0.5 – 0.6 was reached. The cells were then exposed to a bile shock by adding 0.5 % (w/v; final concentration) porcine bile. Following 20 minutes bile exposure cells were harvested by centrifugation, while a culture in fRCM to which no porcine bile was added was also harvested as a control. RNA extraction was carried out as previously reported but with modifications (69). In order to extract RNA, total RNA of each of the cultures was mixed with 800 µl of QIAzoL Lysis Reagent (Qiagen, UK) and pipetted in to a sterile tube with glass beads (Merck, Germany). Cells were lysed 2 minutes of stirring this mixture in a Precellys 24 homogenizer (Bertin instruments, France) with 2 minutes of static cooling; this step was repeated in triplicate. The lysed cells were centrifuged to remove cellular debris at 12,000 rpm for 15 min and the upper phase was collected. The RNA samples were purified using the RNAesy Mini Kit (Qiagen, UK) as per the manufacturer's protocol. RNA concentration and purity were checked by a Picodrop microliter spectrophotometer (Picodrop, UK).

RNAseq analysis performed by NextSeq Illumina. RNAseq analysis was carried out as previously described with modifications (70). A total of 2.5 µg of RNA was treated to remove ribosomal RNA by the Ribo-Zero Magnetic Kit (Illumina) for RNA sequencing, and the rRNA-depleted sample purified by ethanol precipitation. RNA was processed according to the manufacturer's protocol. The yield of rRNA depletion was measured by a Tape station 2200 (Agilent Technologies, USA). The construction of the whole transcriptome library was carried out using the TruSeq Stranded RNA LT Kit (Illumina). Samples were loaded into a NextSeq High Output v2 Kit Chemicals 150 cycles (Illumina) as per the technical support guide. The reads were depleted of adapters, quality filtered (with overall quality, quality window and length filters) and aligned to the *B. breve* UCC2003 genome.

Inhibition and Dispersal assays. In order to study the factors involved in the initial steps of biofilm formation, an inhibition assay was performed as previously described (54) with some modifications as follows. RCM supplemented with 0.5 % (w/v) porcine bile was inoculated with 10 % overnight *B. breve* UCC2003 wildtype (WT) strain and *B. breve* UCC2003::Bbr_430 (EPS^--negative phenotype) (51). The RCM was also supplemented with 0.95 mg/ml proteinase K (Sigma Aldrich),10 U/µl DNase1 (Sigma Aldrich) or 4 mM sodium (meta) periodate (Sigma Aldrich). Cells were left to form biofilm anaerobically for 24 Hrs at 37ºC, after which biofilm biomass was stained with crystal violet as described above. To investigate what mature biofilm biomass is composed of, biofilm was allowed to form for 24 Hrs as for the inhibition assay and treated as previously described with modifications (71). The planktonic phase was removed, and biofilms treated with 0.95 mg/ml of proteinase K in 20 mM Tris-HCl or 5 U/µl of DnaseI in 5 mM $MgCl_2$ for a further 24 Hrs at 37ºC anaerobically. Biofilms were then stained with crystal violet as stated above.

Viability Assays. To access the viability of cultures after 24 Hrs growth in bile, overnight cultures of bifidobacteria were inoculated as above for biofilm assays into either RCM supplemented with 0.5 % (w/v) porcine bile or RCM only, as an untreated control, and incubated for 24 Hrs. After this culture medium was diluted in PBS and spot plated onto RCA. Plates were incubated for 48 Hrs anaerobically at 37ºC. Cultures were also grown in glass test tubes in the presence of 0.5 % (w/v) bile and allowed to form biofilm for 24 Hrs. Biofilm was then washed three times with sterile water and a pipette tip was used to scrape biofilm from the surface of the test tube. Biofilm was then restreaked on RCA supplemented with 0.05 (v/v) % cysteine-HCl (Sigma) and 0.3 % lactose (Sigma).

5.4. Results

Biofilm induction in Bifidobacteria.

Bifidobacteria may encounter various stresses in the GIT such as acid and bile salt stress (17). In other bacterial species, acid stress (45), salt stress (46) and bile exposure are known to induce biofilm formation (47). Bile salt (and by inference bile itself) concentrations vary along the GIT between 1 mM and 40 mM (21). Therefore, we tested various conditions, using an established method for biofilm detection, the crystal violet assay, to investigate under what conditions biofilm formation occurs in bifidobacteria. Previously, biofilm formation by various bifidobacterial species had been detected by means of congo red and crystal violet staining assays, and shown to occur following exposure to 0.5 % taurocholic acid and porcine bile at 5 % (w/v) (34). As expected, and using the prototype bifidobacterial gut commensal *B. breve* UCC2003 it was shown that biofilm formation indeed occurs following (porcine) bile exposure. However, because bile concentrations fluctuate throughout the GIT, we wanted to assess if biofilm formation is induced by other conditions pertinent to the intestinal environment and to what extent this occurs by varying porcine bile concentrations **(Figure 5.1)**. Our findings show that biofilm formation is not induced in *B. breve* UCC2003 by low pH or osmotic stress (NaCl or sucrose) as has been reported for other bacterial species (46). All tested bile concentrations were considered physiologically relevant, and the biofilm-inducing effect of porcine bile was clearly shown to be dose dependent. Under the conditions tested biofilm formation by *B. breve* UCC2003 did not occur to any appreciable extent at bile concentrations of 0.05 % and 0.1 % (w/v), whereas at higher bile concentrations, i.e. 0.5 % and above, clearly detectable biofilms were formed by this strain (**Figure 5.1A**). Of note, addition of porcine bile to the RCM media did not cause a change in pH, and we therefore presume that the induction of biofilm formation is pH independent. Furthermore, we tested several bifidobacterial species/strains to assess if dose-dependent, bile-induced biofilm formation is exhibited by other members of the bifidobacterial genus. All examined bifidobacterial strains/species were indeed shown to produce a biofilm in the presence of 0.5 % or 1 % (w/v) porcine bile **(Figure 5.1B)**. Therefore, biofilm formation

in the presence of high concentrations of bile seems to be a property elicited by multiple species/strains across the genus *Bifidobacterium*.

Bile is a heterogeneous mix of various components including cholesterol, bile salts, proteins and bilirubin (20). We therefore wanted to assess if bile salts alone are capable of inducing biofilm formation. Both taurine and glycine primary bile salts were tested along with their dehydroxy derivatives to see if any particular type of bile salt acts as a specific inducer for this process. Using *B. breve* UCC2003 it was shown that biofilm formation was triggered by individual bile salts **(Figure 5.2)** and that biofilm formation typically occurs at higher concentrations of bile salts, i.e. 20 mM and 40 mM, while at lower concentrations, i.e. 1 mM and 10 mM, very little or no biofilm was observed. Both trihydroxy-conjugated bile salts, taurocholic acid (TC) and glycocholic acid (GC), and dihydroxy-conjugated bile salts such as taurodeoxycholic acid (TDC), chenodeoxycholic acid (CDC) or glycodeoxycholic acid (GDC) induced biofilm formation **(Figure 5.2)**. Therefore, biofilm formation by bifidobacteria upon exposure to bile/bile salts is a common phenomenon and may represent an adaptation mechanism to specifically survive exposure to high levels of bile encountered in the GIT.

Transcriptomic response of *Bifidobacterium breve* UCC2003 to a high concentration of bile.

In order to determine the transcriptomic response of *B. breve* UCC2003 to a high concentration of bile and to assess whether these genes were implicated in biofilm formation (see below), this strain was cultivated to a corresponding $O.D._{600nm}$ value between 0.5 and 0.6, and then exposed for twenty minutes to 0.5 % ((w/v) final concentration) porcine bile. Genes exhibiting transcriptional upregulation/downregulation following bile exposure, with an associated *p*-value of less than 0.05, are summarised in **Table 5.3**.

Various genes predicted to be involved in transport and metabolism of carbohydrates were significantly upregulated following 0.5 % bile exposure. Transcription of a gene encoding a putative PEP-PTS system (Bbr_1594), which

previously was shown to be induced by glucose (48), was upregulated 6-fold under the imposed bile exposure conditions. Similarly, genes predicted to encode an ABC-type transporter permease (Bbr_1558), an ATP-binding protein for an ABC-type transporter (Bbr_1890), galactokinase (Bbr_0492), acetate kinase (Bbr_0771) and xylulose-5-phosphate/Fructose-6-phosphate phosphoketolase (Bbr_0776) were shown to be transcriptionally upregulated under these conditions, indicating that carbohydrate uptake and active carbohydrate metabolism are associated with the adaptive response to bile stress. However, our results also show that transcription of other genes involved in transport and metabolism of carbohydrates was subject to downregulation upon exposure to bile. These included an ATP-binding protein of an ABC-type transporter system (Bbr_0808), a glucosamine 6-phosphate isomerase (Bbr_1248), a cellodextrin binding protein (Bbr_0106), 1-4 α glucan branching enzyme and others summarised in **Table 5.3**. Therefore, it seems that a specific response of increased carbohydrate metabolism is induced following the imposition of bile stress.

A solute binding protein (SBP) of an ABC-type transporter (Bbr_0521) of the bac3 family possibly involved with glutamate and histidine uptake was also downregulated. A presumed SBP (Bbr_0579) implicated involved in Zn/Mn transport and previously found to be upregulated under iron limitation conditions (49), was downregulated 31 fold. Transcription of genes predicted to be involved in polyketide synthesis (Bbr_0204/0205)/ fatty acid metabolism (Bbr_1719) also incur upregulation in response to bile shock. Other genes, whose transcription was shown to increase upon bile exposure, were predicted to be involved in cysteine metabolism (Bbr_0969), ATP production (Bbr_328/329), iron-sulfur metabolism (Bbr_0911) and an ATP component of the oligopeptide nucleotide transporter OppD (Bbr_1202).

Whether or not the genes involved in bile resistance and genes involved in biofilm formation are interconnected remains to be seen. Therefore, we decided to investigate which genes are involved in biofilm formation and to determine if these genes are akin to the genes upregulated in the shock exposure to bile.

Screening of a transposon-mediated insertion mutant library of *B. breve* UCC2003.

In order to identify genes involved in biofilm formation, a previously described transposon mutant library of *B. breve* UCC2003 (49, 50) was screened for mutants affected in biofilm formation. Biofilm induction was achieved employing exposure of individual mutants to high concentrations of porcine bile, 0.5 or 1 % (w/v), for 24 Hrs; biofilm biomass was stained using crystal violet, solubilised in acetic acid and an associated $O.D._{570nm}$ measurement was taken to perform a semi-quantitative assessment of biofilm biomass. A reduced $O.D._{570nm}$ value (compared to that obtained for the wild type strain *B. breve* UCC2003) indicated a reduction in biofilm biomass formation and suggested that the transposon had mutated a gene involved in biofilm formation. A positive control of *B. breve* UCC2003 and transposon mutants grown in RCM was also included to exclude mutants that were simply impaired in growth (OD_{600nm} value being < 0.5) which could have reduced biofilm biomass because of reduced cell numbers due to poor growth. The screen was carried out with RCM to prevent identifying mutants defective in growth of a single carbon source (as RCM contains both glucose and starch). Transposon mutants identified in the primary screen where retested in a confirmatory screen in triplicate to ensure no false positives were isolated. 10,000 transposon mutants were screened from the *B. breve* UCC2003-derived transposon library, resulting in the identification of eleven mutants that were shown to be clearly and consistently affected in their ability to form a biofilm (**Table 5.4; Supplementary Figure S5.1)**.

The location of the transposon in individual mutants was identified by direct inverse PCR (iPCR) amplification or arbitrary primed PCR and subsequent sequencing (see Materials and Methods) and predicted gene functions were assigned by BlastP analysis. Alongside these mutants two other previously described mutants in a gene encoding a predicted priming glycosyl hydrolase causing loss of EPS production (EPS^-) and a mutant in the gene for the AI-2-producing LuxS enzyme were also tested (43, 51). All eleven identified transposon and the two additionally selected mutants tested exhibited reduced biofilm biomass compared to *B. breve* UCC2003 WT at 24 Hrs as determined by the crystal violet assay **(Figure 5.3)**. The *B. breve* UCC2003 EPS^- mutant was shown to elicit substantially reduced biofilm biomass as compared to the wildtype suggesting that

EPS is important in biofilm formation. Several genes involved in metabolism and physiology where found to be involved in biofilm formation, such as (i) *nrdHIE*, which encodes a ribonucleotide reductase, (ii) SerA2, a phosphoglycerate dehydrogenase/ thymidlate synthesis, (iii) Bbr_200, an NADH flavin reductase, (iv) Bbr_200, an AAA ATPase, and (v) *glgP*, a glycogen phosphorylase, which is an enzyme responsible for the breakdown of glycogen (52). Transposon-mediated disruption of genes that influence the composition and properties of the cell wall envelope also had an impact on biofilm formation, such as *dapE*. DapE is a N-succinyl-L,L-diaminopimelic acid desuccinylase part of the lysine/*meso*-diaminopimelate (mDAP) pathway that produces lysine for protein synthesis and both lysine and mDAP are required for peptidoglycan synthesis (53). A mutant in a gene responsible for type I fatty acid biosynthesis (*fas*) also exhibited reduced biofilm biomass. A total of three transposon mutants in *accC* were isolated from the transposon bank screen. The *accC*, *accD* and *fas* genes, putatively encoding the acetyl Co-A α chain, acetyl Co-A β chain and the fatty acid synthase enzymes, respectively, are adjacent to each other and mutations in these genes are believed to interfere with fatty acid biosynthesis. Furthermore, mutations in genes involved in amino acid metabolism, such as a predicted oligopeptide transporter OppD2 and a predicted peptidase PepX, were shown to affect biofilm formation.

From the above mutant screen, it is apparent that biofilm formation is a complex process involving a diverse set of genes involved in, among others, EPS production, in S-ribosylhomocysteinase production, as well as carbon, fatty acid and peptide metabolism. Some of the genes, such as Bbr_1719; involved in fatty acid synthesis (*accC*), and Bbr_1202 (*oppD*) found in the screen were also upregulated in response to high concentrations of bile. Therefore, we wondered if biofilm was a survival strategy in response to high concentrations of bile.

Biofilm viability.

Biofilm formation seems to be associated with bile resistance and in order to investigate if this biofilm forming ability is positively correlated with enhanced survival following bile exposure, *B. breve* UCC2003 WT, the *luxS* insertion mutant, the EPS$^-$

mutant, as well as transposon mutants in *accC* and Bbr_201 were grown for 24 Hrs in RCM supplemented with (0.5 %; w/v) or without porcine bile. Culture media was then diluted in PBS and spot plated on RCA to determine viable counts. Under these conditions *B. breve* UCC2003 WT and *accC* mutant were shown to exhibit the highest survival level compared to any of the other mutants (**Figure 5.4A)**. To test if the biofilm formed was viable after 24Hrs, these strains were also grown in test tubes in the presence of porcine bile (0.5 %; w/v) and left for 24 Hrs to allow biofilm to form. Biofilm was then scraped off with a pipette tip and restreaked on RCA supplemented with cysteine and 0.5 % lactose **(Figure 5. 4B)**. The RCA plates were then incubated for 48 Hrs and any colonies present counted. Viable colonies could be recovered from biofilm of *B. breve* UCC2003 WT and for all the mutants even though these mutants had less biofilm biomass. Therefore, these results suggest that the biofilm biomass itself is viable and that biofilm formation can increase resistance to high concentrations of bile.

Biofilm matrix composition.

In other bacterial species, cell wall associated proteins, EPS and eDNA are involved in the initiation and accumulation stages of biofilm (46, 54, 55). Therefore, in order to get an insight into the initiation stages of biofilm formation, biofilms for *B. breve* UCC2003 wildtype were set up in microtiter plates as above but were also incubated with proteinase K, to degrade proteins, or DNaseI, to degrade eDNA, and sodium metaperiodate, to oxidise EPS/cell surface carbohydrates, in order to assess if proteins, eDNA or extracellular surface carbohydrates play a role in (the initial stages of) biofilm formation **(Figure 5.5)**. Incubation with proteinase K, DNaseI and sodium (meta) periodate was shown to cause a reduced biofilm biomass after 24 Hrs as indicated as a reduced $O.D._{570nm}$ value as compared to untreated *B. breve* UCC2003 WT **(Figure 5.5A)** biofilm suggesting that the attachment and accumulation phases are mediated by a combination of proteins, extracellular DNA release and carbohydrate secretion, presumably EPS mediated. This indicates that macromolecules such as cell wall-associated proteins, eDNA and EPS are involved in the initial attachment and accumulation phases of bifidobacterial biofilm formation. The *B. breve* UCC2003 EPS-

negative mutant appeared to produce substantially less biofilm biomass than the WT, and extended treatment with DNaseI and proteinase was shown to reduce biofilm yet did not abolish biofilm completely. Treatment with sodium (meta) periodate did substantially reduce biofilm formation suggesting perhaps other cell wall-associated polysaccharides are important in biofilm formation. In the latter context it is relevant to note that *B. breve* UCC2003 has been reported to contain two EPS clusters (51, 56).

To investigate the composition of the EM of mature biofilms of the *B. breve* UCC2003 WT formed after 24 Hrs, biofilms were enzymatically treated with proteinase K and DNaseI to determine if protein and/or DNA contributed to the EM, respectively **(Figure 5.6)**. Proteinase K was able to disperse mature biofilm of *B. breve* UCC2003 WT, whereas DNaseI could not. This suggests that while extracellular DNA release may be important in the initial stages of biofilm formation it may not be as important in established mature biofilm structures. Proteinase K could also not completely disperse biofilm in *B. breve* UCC2003 WT, suggesting that mature biofilm composition is a multifactorial process, involving multiple macromolecules. In fact, complete (mature) biofilm dispersal was only observed when the *B. breve* UCC2003 EPS^- mutant was treated with proteinase K. This suggests that both EPS and proteins play an important role in mature biofilm formation.

5.5. Discussion.

Bifidobacteria are gut commensals and to survive in the GIT environment they must be able to survive bile exposure. Our findings show that bifidobacteria form a biofilm following exposure to high concentrations of porcine bile. Porcine bile possesses a glycine:taurine ratio which is similar to that of human bile (57). Previous studies characterising the bifidobacterial bile response used bovine bile, rather than porcine bile, while also employing bile/bile salts at lower concentrations than those shown to induce biofilm formation (24, 25, 28, 29). It is important to assess the bifidobacterial response to various concentrations of bile as there is a gradient of bile in the GIT. The transcriptomic

response of *B. breve* UCC2003 to a high concentration (i.e. 0.5 % w/v or higher) of porcine bile was also distinct from bile exposure to lower concentrations of bile, oxgall 0.15 % (w/v) and cholate 0.06 % (w/v), as previously reported (24). The transcriptomic response of *B. breve* UCC2003 to a high concentration of bile was shown to involve specific response in carbohydrate metabolism. This is in agreement with previous proteomic studies assessing bile response, where the expression of glycolytic enzymes and pyruvate catabolism enzymes, such as acetate kinase and xylulose-5-phosphate/fructose-6-phosphate phosphoketolase, was upregulated (29, 30, 58). Bile-adapted bifidobacterial strains have a different carbohydrate preference as compared to WT strains (59). Therefore, bile shock seems to invoke specific changes in carbohydrate uptake, storage and metabolism that may be important to survive high bile concentrations.

Genes involved in bile resistance also seem to be connected to biofilm formation. Our findings show that on exposure to high concentrations of bile fatty acid biosynthesis is induced, which also contributed to biofilm formation. A mutant in *accC* was shown to elicit increased resistance to bile, which suggests that fatty acid synthesis is not only important for biofilm formation but also for bile resistance. Previous studies have reported that transcription of the fatty acid synthase genes is downregulated when bifidobacteria are exposed to bile (24, 27). However, these studies were conducted at lower concentrations of bile with either bovine bile and/or individual bile salts rather than porcine bile and this may explain this apparent discrepancy. It is unknown why fatty acid metabolism is important in bile resistance. It has previously been shown that bile induces biofilm formation due to its capacity to increase surface hydrophobicity of bifidobacterial cells (34). Therefore, changes in surface hydrophobicity and perhaps membrane permeability due to altered fatty acid synthesis may help to resist the bacteriocidal effects of bile. Similarly, OppD2 was shown to be upregulated and involved in biofilm formation. It has previously been reported that OppA production is upregulated in bifidobacteria upon exposure to bile and shown to allow increased uptake of oligopeptides (24, 60). Oligopeptide transporters have also been implicated in bile resistance in *Lactobacillus salivarius* (61), although the precise manner by which peptides are involved in biofilm formation and bile resistance is currently not clear.

Bifidobacteria have been shown to form biofilm in the GIT environment (37, 38). We identified various genes involved in biofilm formation and we have shown that some of the corresponding mutants exhibit reduced viability following growth in bile. The *luxS* mutant was previously shown to impact on GIT colonisation in a mouse model (43). Similarly, insertion in *luxS* has an impact on biofilm formation and colonisation persistence in lactobacilli (62). However, the effect of a *luxS* insertion was not found to be exclusively due to absence of AI-2 production, but due to specific metabolic effects, such as changes in fatty acid metabolism and cysteine/sulfur-containing amino acid metabolism (36, 63). Genes involved in cysteine synthesis were upregulated in *B. breve* UCC2003 under shock with 0.5 % (w/v) porcine bile. LuxS is responsible for bifidobacterial synthesis of AI-2, yet bifidobacteria appear to lack an AI-2 quorum sensing system such as LuxP and/or LsrB, and we can therefore only speculate as to the mechanism by which AI-2 production is linked to bile resistance (43, 64). The *B. breve* UCC2003 EPS^- mutant has also been shown to be less resistant to 0.3 % (w/v) porcine bile, while eliciting a reduced colonisation persistence in the GIT of mice (51).

We also show that biofilm formation requires different macromolecular factors: the initial attachment phase of biofilm seems to be dependent on eDNA, EPS and protein interactions, though eDNA does not appear to be as important in the mature biofilm structure. A limitation of our study is that we could not distinguish if genes were important for initiation or maturation phases due to the screen being carried out in microtiter plates. More investigation is thus needed to dissect which genes are important for each of the phases of biofilm development and to discern if the importance of *luxS* in biofilm is due to AI-2 production or metabolic changes.

From our study we propose the following model of biofilm in bifidobacteria in response to high concentrations of bile based on our works findings and biofilm in the literature **(Figure 5.7)**. High concentrations of bile (0.5 % and above) lyse bifidobacterial cells and may release intracellular signals such as AI-2 or oligopeptides to induce quorum sensing. Extracellular DNA released from lysed cells may also coat the surface and resulting additional electrostatic interactions that allow bifidobacteria to adhere. Bile increases hydrophobicity of the cell surface and allows initial attachment of bifidobacteria

to the surface by increased hydrophobic interactions with the surface. Increased fatty acid biosynthesis may also alter cell surface membrane properties and LuxS may produce metabolic changes to also alter the cell membrane composition. Secretion of EPS and protein interactions may then allow firmer attachment and accumulation of cells. Maturation of the extracellular matrix of the biofilm involves further EPS secretion and protein interactions. When high concentrations of bile decrease, the biofilm may disperse and bifidobacterial cells are free to grow planktonically again. Future studies will be needed to test this model for accuracy, while additional studies are also needed to determine how important biofilm formation is for bifidobacterial gut colonisation and survival in specific parts of the GIT.

5.6. Author contributions.

S.M.K. designed experiments, carried out experiments, interpreted results and wrote the manuscript. N.L. designed experiments and carried out experiments. I.O.N. designed experiments. F.B. interpreted results. G.A.L., A.V., F.T., carried out experiments. M.V. designed experiments. D.v.S. designed the experiments, interpreted results and wrote the manuscript.

5.7. Acknowledgements.

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5.8. Tables and Figures

Table 5.1.Strains and plasmids used in this work.

Bacterial Strain/ plasmid	Features	Reference
Bifidobacterium breve		
UCC2003		(48)
UCC2003::Bbr_430	Insertional mutant in Bbr_430 gene of the EPS cluster.	(51)
UCC2003-luxS	Insertion mutant in *luxS* – (Bbr_0541)	(43)
JCM 7017		
JCM 7019		
NCTC 11815		
Bifidobacterium longum **subsp. *longum***		
NCIMB 8809		
CCUG 30698		
Bifidobacterium longum **subsp. *infantis***		
ATTC 15697		
Bifidobacterium dentium		
DSM 20436		
Bifidobacterium adolescentis		
DSM 20083		
Bifidobacterium pseudolongum		
DSM 20438		

Table 5.2.Oligonucleotides used in this study

Primer	Function	Sequence
iPCR-Fw	Forward primer for inverse PCR reaction	GCATACCGTACTGATCTG
iPCR-Rv	Reverse primer for inverse PCR reaction	CAATCATACCGGCTTCC
Arb6	Primer for arbitrary PCR	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC
Arb2	Primer for arbitrary PCR	GGCCACGCGTCGACTTAGTTAC
Arb1	Primer for arbitrary PCR	GGCCACGCGTCGACTAGTTACNNNNNNNNNNGATAT
TnTetL1	Primer for arbitrary PCR	AAAACATGGTGTCCGTCCTC
TnTetR1	Primer for arbitrary PCR	TCGCTGGGATACTTGAACCA
TnTetL2	Primer for arbitrary PCR	GCTGTGGTGTTTGGTTGGAA
TnTetR2	Primer for arbitrary PCR	CTCTATGCGCCCCAGGAATA
pMod-Fw-seq	Forward sequencing primer based on transposon	GCCAACGACTACGCACTAGCC
pMod-Rv-seq	Reverse sequencing primer based on transposon	GAGCCAATATGCGAGAACACC

Table 5.3. Genes transcriptionally upregulated or downregulated in response to 0.5 % (w/v) porcine bile.

Locus tag	Putative Function	Fold change in expression (Up regulation)	P -value
Bbr_0376	Hypothetical protein	10.08	9.18E-14
Bbr_1594	PTS system fructose/glucose (*fruA*)	6.09	4.99E-15
Bbr_0925	Permease MFS superfamily	5.49	0.000125407
Bbr_0204	Multi-domain protein fatty acids or polyketide synthesis	4.33	1.79E-10
Bbr_1558	Permease of ABC transporter	4.16	1.98E-06
Bbr_0205	Multi-domain protein fatty acids or polyketide synthesis	3.81	2.43E-08
Bbr_0521	Solute binding protein of ABC transporter, bac 3 family.	3.76	1.65E-07
Bbr_1890	ATP binding protein for ABC transporter	3.67	4.95E-09
Bbr_0492	Galactokinase	3.52	0.004216372
Bbr_0188	Formate – tetrahydrofolate ligase	3.00	0.000253137
Bbr_1719	Type I Multi-functional Fatty Acid Synthase	2.78	1.07E-07
Bbr_1615	DNA- directed RNA Polymerase Alpha Chain	2.38	5.34E-06
Bbr_1010	HIT Family Hydrolase	2.35	0.004013209

Bbr_1638	RplB, 50S ribosomal L2 protein	2.05	3.04E-05
Bbr_0183	Guanine-hypoxanthine permease	2.04	8.92E-05
Bbr_0911	IscU – Iron sulfur scaffold protein	1.93	0.000509005
Bbr_1002	Transaldolase	1.87	8.14E-07
Bbr_0499	DNA-directed RNA polymerase beta' chain	1.79	1.65E-05
Bbr_0969	Homocysteine methyltransferase	1.67	0.000468744
Bbr_0377	Oxidoreductase aldo/keto reductase family	1.63	0.003458674
Bbr_0771	Acetate kinase	1.52	0.000906418
Bbr_0329	ATP synthase beta chain	1.42	0.000860229
Bbr_0328	ATP synthase gamma chain	1.41	
Bbr_1202	Oligopeptide transport ATP-binding protein oppD	1.28	0.001120451
Bbr_0371	Polyribonucleotide nucleotidyltransferase	1.23	0.000351287

Bbr_0776	Xylulose-5-phosphate/Fructose-6-phosphate phosphoketolase	1.13	0.00154578

Locus tag	**Putative function**	**Fold change in expression (downregulation)**	**P -value**
Bbr_0579	Solute binding protein of ABC transporter system, iron siderophore, metallic cations (Zn/Mn transport)	31.39	4.25E-39
Bbr_0808	ATP-binding protein of ABC transporter system	30.33	4.51E-15
Bbr_0538	Cysteine synthase	12.64	2.48E-06
Bbr_1354	Transciptional regulator	12.16	0.004471376
Bbr_0849	NagC/XylR-type transciptional regulator	11.58	2.80E-08
Bbr_0008	Transcriptional regulator LacI family	11.01	0.002749448
Bbr_1248	Glucosamine-6-phosphate isomerase	9.41	0.000796378
Bbr_1860	Solute binding protein of ABC transporter system for sugars	9.15	0.004306336
Bbr_0083	(Filamentation induced by cAMP) Fic family protein	9.04	0.00351993
Bbr_1791	Phosphoglycerol transferase	7.13	5.68E-05

Bbr_1781	ClpB protein	6.59	3.08E-07
Bbr_1506	Cyclopropane-fatty-acyl-phospholipid synthase	5.84	2.74E-05
Bbr_1793	ATP-binding protein ABC transporter system for polysaccharides	4.55	0.000502173
Bbr_0751	Solute-binding protein of ABC transporter system for metals	4.42	0.000817759
Bbr_1590	Solute-binding protein of ABC transporter system for sugars	4.26	3.50E-08
Bbr_0106	Cellodextrin binding protein	4.17	0.000211367
Bbr_0348	Aspartate ammonia-lyase	4.06	0.005724464
Bbr_0070	Cell division protein FtsW	3.89	0.003286088
Bbr_1251	N-acetylglucosamine repressor	3.78	0.005702581
Bbr_0417	Solute-binding protein of ABC transporter system for sugars galactan metabolism	3.37	4.61E-05
Bbr_1790	Phosphoglycerol transferase	3.26	0.003699621
Bbr_0027	Permease protein of ABC transporter system for sugars	3.17	0.000263903

Bbr_0809	Permease protein of ABC transporter system	2.15	0.005541407
Bbr_1889	Cell surface protein with gram positive anchor domain	1.98	0.005000633
Bbr_0746	14-alpha-glucan branching enzyme	1.82	0.002004982
Bbr_1574	Phosphoglycerate mutase	1.79	0.00071798
Bbr_1710	Ribokinase	1.75	0.001314349

Table 5.4.Transposon insertions isolated in crystal violet biofilm screen

Mutant	Gene locus	Function
Bbr_1202*	*oppD2/oppB2/ oppC1*operon.	Oligopeptide transporter
Bbr_1738	*dapE*	Succinyl-diaminopimelate desuccinylase, lysine and cell wall synthesis
Bbr_1901	*nrdH*, *nrdI*, *nrdE* operon	Ribonucleotide reductase
Bbr_0074/0075	*pepX*	Xaa –Prolyl Peptidase
Bbr_1719/20/21†	*accC/accD/fas* operon	Fatty acid biosynthesis
Bbr_200	*NADH Flavin reductase*	DNA binding protein/NADH Flavin reductase
Bbr_201		DNA binding protein/AAA ATPase
Bbr_1654/53/52/51	*serA2*	Non-functional conserved protein/Phosphoglycerate dehydrogenase/Thymidlate synthase
Bbr_0060	*glgP1*	Glycogen phosphorylase
Bbr_1353	*proP*	Osmolarity/stress MFS
Bbr_1580		Transmembrane protein/hydrolase

***Gene was isolated twice in mutant screen. (Distinct mutants in the same gene).**

†Gene was isolated three times in mutant screen. (Distinct mutants in the same gene).

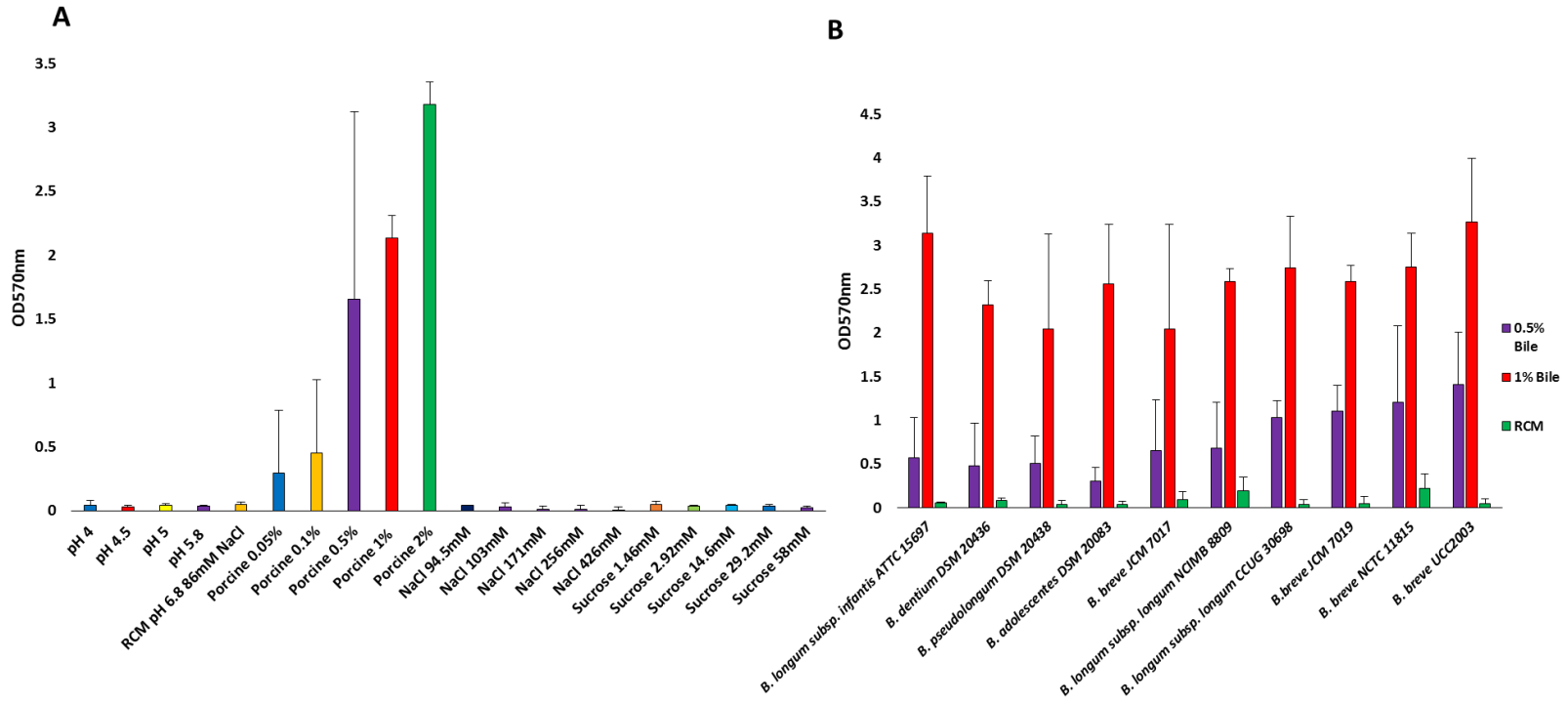


Figure 5.1. Biofilm formation by *Bifidobacterium breve* UCC2003 under different stress conditions.

Biofilm was allowed to form for 24 Hrs under various conditions including pH 4 – 6.8, sucrose 0.05 mM to 2 mM, NaCl 0.05 mM to 2 mM and porcine bile 0.05 % to 2 % (w/v) **(A)**.. The pH of RCM was 6.8. Biofilm formation was assessed by crystal violet staining with absorbance read at $O.D._{570nm}$. Biofilm formation by several species/strains of bifidobacteria. Biofilm induced by addition of 0.5 % or 1 % (w/v) of porcine bile and allowed to form for 24 Hrs **(B)**. Biofilm was stained with crystal violet and the absorbance read at $O.D._{570nm}$. Negative controls with just RCM (non-inducing biofilm conditions) were also included for each species. Experiments were carried out in triplicate and error bars represent standard deviation.

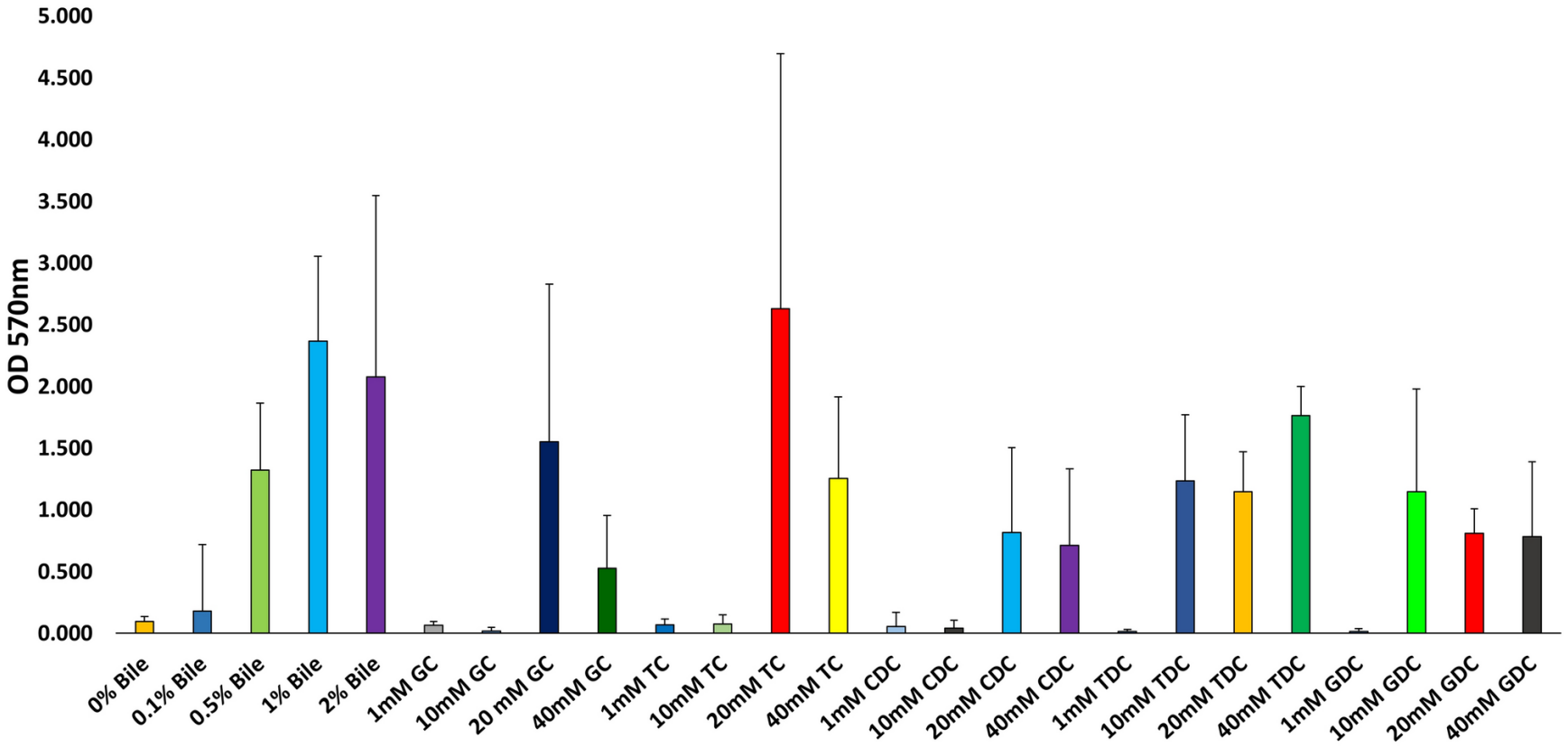


Figure 5.2. Biofilm formation of *Bifidobacterium breve* UCC2003 in response to bile salts.

Biofilm formation was induced by addition of glycocholic acid (GC), taurocholic acid (TC), chenodeoxycholic acid (CDC), taurodeoxycholic acid (TDC) and glycodeoxycholic acid (GDC) at concentrations of 1 mM, 10 mM, 20 mM and 40 mM. Biofilm was allowed to form for 24 Hrs, was stained using crystal violet and the absorbance read at $O.D._{570nm}$. Experiments were carried out in triplicate and error bars represent standard error of the mean.

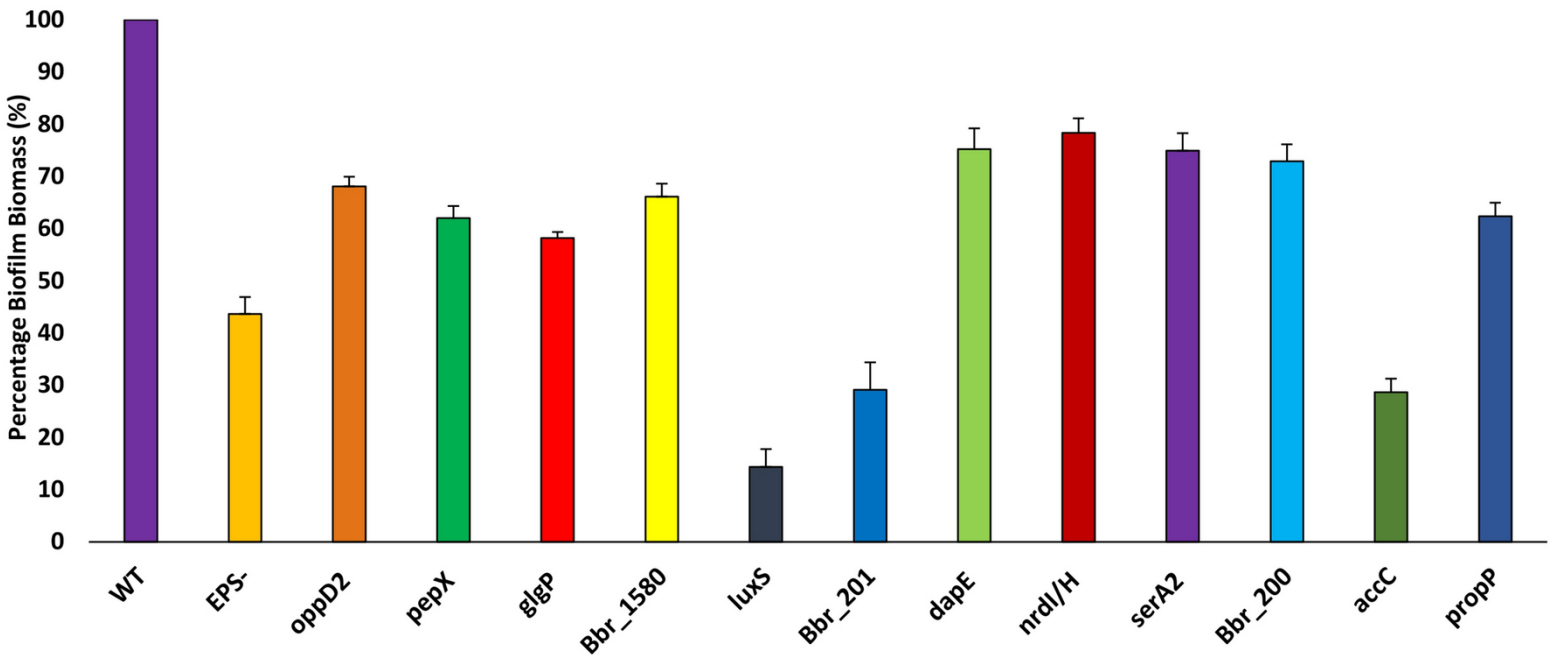


Figure 5.3. Biofilm formation by mutants screened from a *Bifidobacterium breve* UCC2003 transposon mutant bank.

A transposon mutant bank was screened using the crystal violet assay. Biofilm was induced with 0.5 % (w/v) porcine bile and allowed to form for 24 Hrs. An insertional mutant *B. breve* UCC2003 *luxS*, and EPS deficient strain, *B. breve* UCC2003 EPS^-, were also screened. A mutant in biofilm formation was assumed to have reduced biofilm biomass, as compared to the wildtype, due to reduced absorbance at $O.D._{570nm}$. Experiments were carried out in triplicate and error bars represent standard error of the mean.

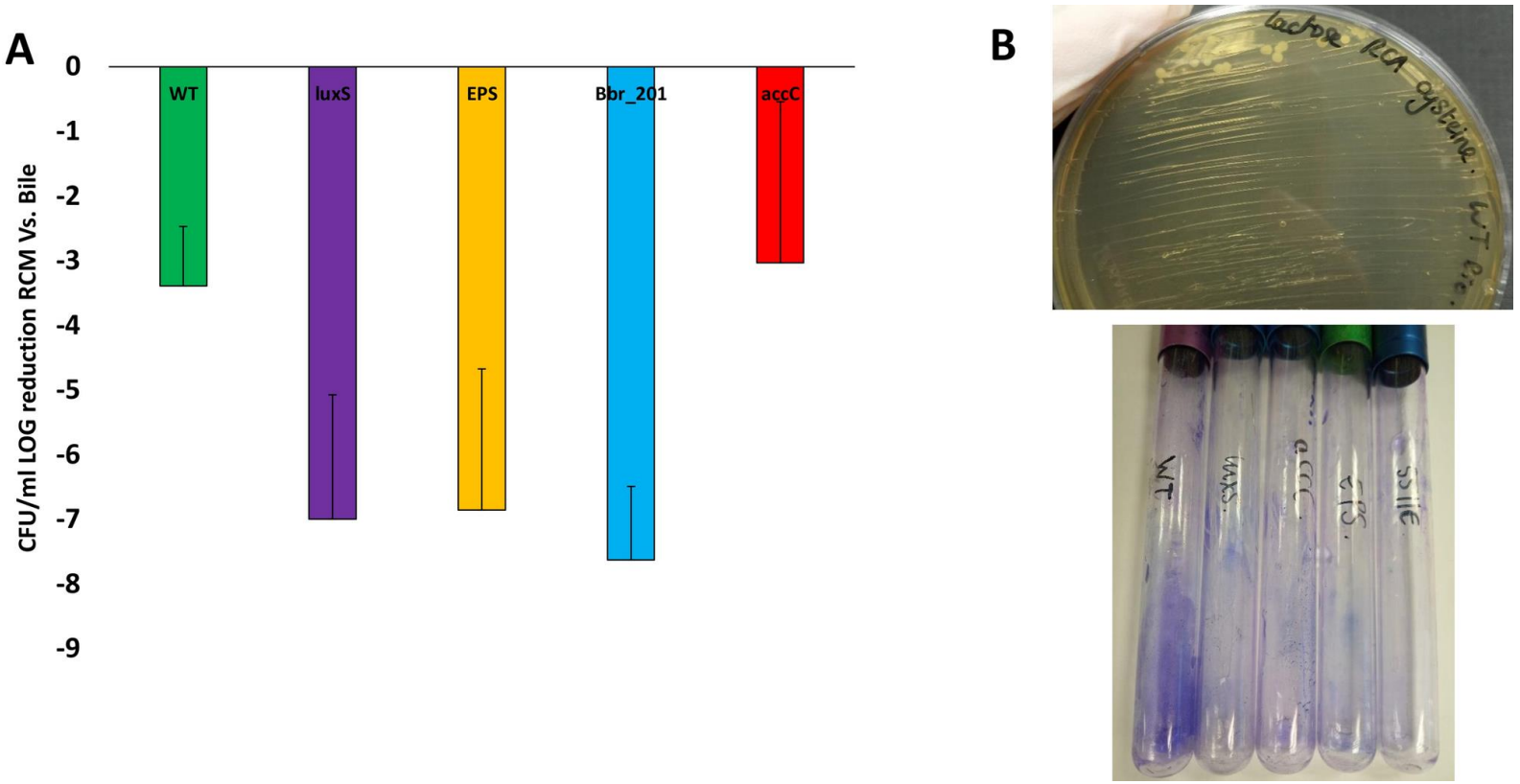


Figure 5.4. Viability of Bifidobacteria after 24 Hrs growth in porcine bile.

B. breve UCC2003 WT, *B. breve* UCC2003 *luxS*, *B. breve* UCC2003 EPS^{-}, *B. breve* UCC2003 *accC* and *B .breve* UCC2003 Bbr_201 were grown in a microtiter plate in RCM supplemented with 0.5 % (w/v) of porcine bile (biofilm formation conditions) and incubated for 24 Hrs. Culture media was then diluted and spot plated on RCA to see if viable bacteria could be recovered and the CFU/ml was calculated **(A)**. Experiments were carried out in triplicate and error bars represent standard deviation. Biofilm was also induced in testubes by growing the above strains in RCM supplemented with 0.5 % (w/v) of porcine bile and incubated for 24 Hrs **(B)**. Supernant was removed and test tubes were washed twice to remove planktonic cells. Biofilm was then scraped off the test tubes, where formed, with a pipette tip and streaked out on RCA supplemeted with lactose and cysteine to obtain viable colony counts (top image). Test tubes were also stained with crystal violet to visualise biofilm (bottom image).

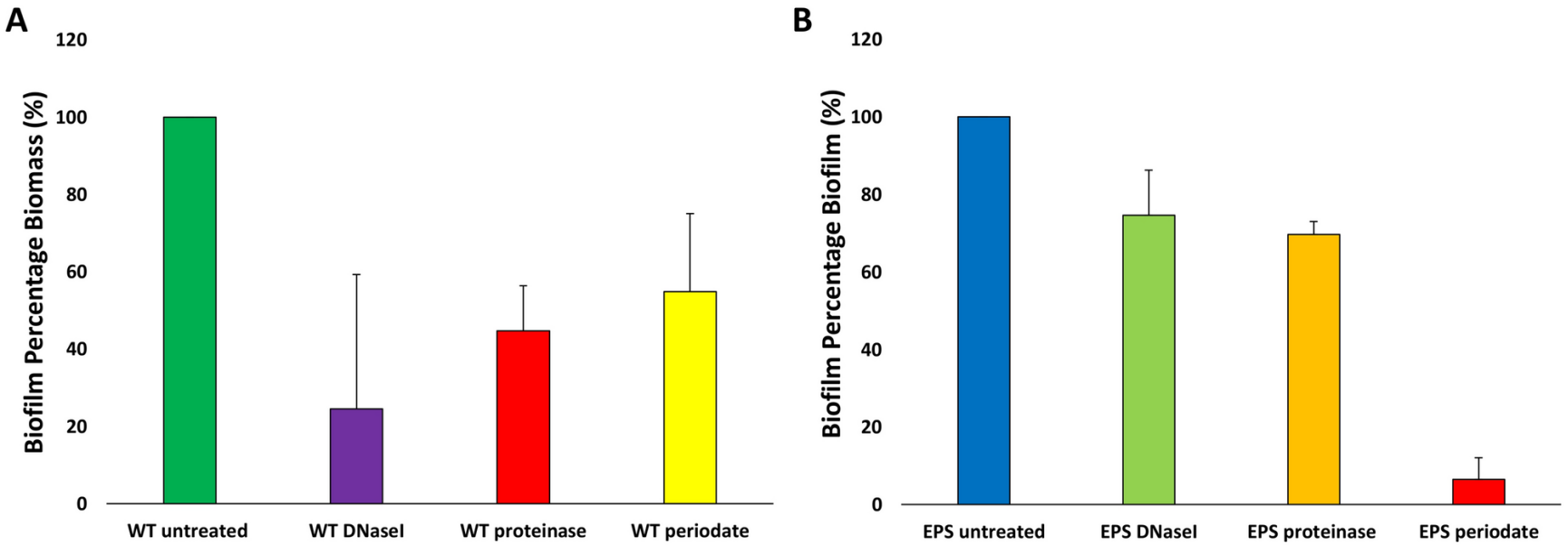


Figure 5.5. Inhibition of biofilm attachment of *Bifidobacterium breve* UCC2003 WT and *Bifidobacterium breve* UCC2003 EPS$^-$

Inhibition of biofilm attachment of *Bifidobacterium breve* UCC2003 wildtype (WT)**(A)** and *Bifidobacterium breve* UCC2003 exopolysaccharide deficient (EPS$^-$)**(B)**. Biofilm was induced by supplementing media with 0.5 % (w/v) porcine bile and was additionally incubated with DNaseI (10 U/ml), proteinase (0.95 mg/ml) or sodium (meta)periodate (4 mM) or left untreated. Biofilms were then left to form for 24 Hrs, biofilm biomass was stained with crystal violet and absorbance read at $O.D._{570nm}$. Maximal biofilm production was taken to be 100 % for *B. breve* UCC2003 WT and *B. breve* UCC2003 EPS$^-$ when comparing effects of DNaseI, proteinase and sodium (meta) periodate on these individual strains. All experiments were carried out in triplicate and errors bars represent standard deviations.

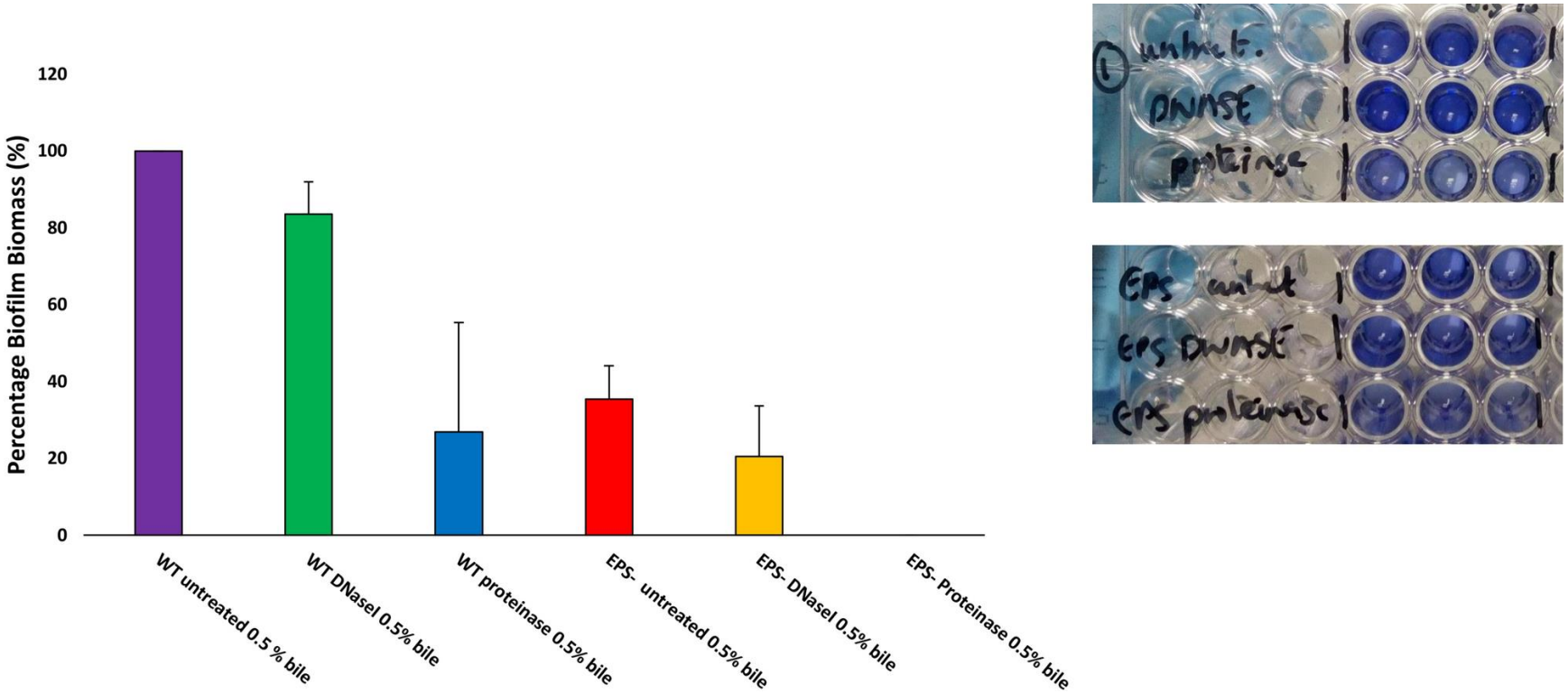


Figure 5.6 Dispersal of mature biofilms of *Bifidobacterium breve* UCC2003 wildtype and *B. breve* UCC2003 EPS^{-}.

Dispersal of mature biofilms of *Bifidobacterium breve* UCC2003 wildtype (WT) and a *B. breve* UCC2003 derivative deficient in exopolysaccharide production (EPS^{-}). Biofilm was induced by supplementation of media with 0.5 % (w/v) porcine bile and biofilms were allowed to form for 24 Hrs. Mature biofilms were then treated with DNaseI (10 U/ml) in 5 mM $MgCl_2$ and 0.95 mg/ml proteinase K in 20 mM in Tris-HCl. Biofilms were stained with crystal violet and absorbance read at $O.D._{570nm}$. Biofilm formation of *B. breve* UCC2003 WT was taken to be 100 %. All experiments were carried out in triplicate and error bars represent standard deviations.

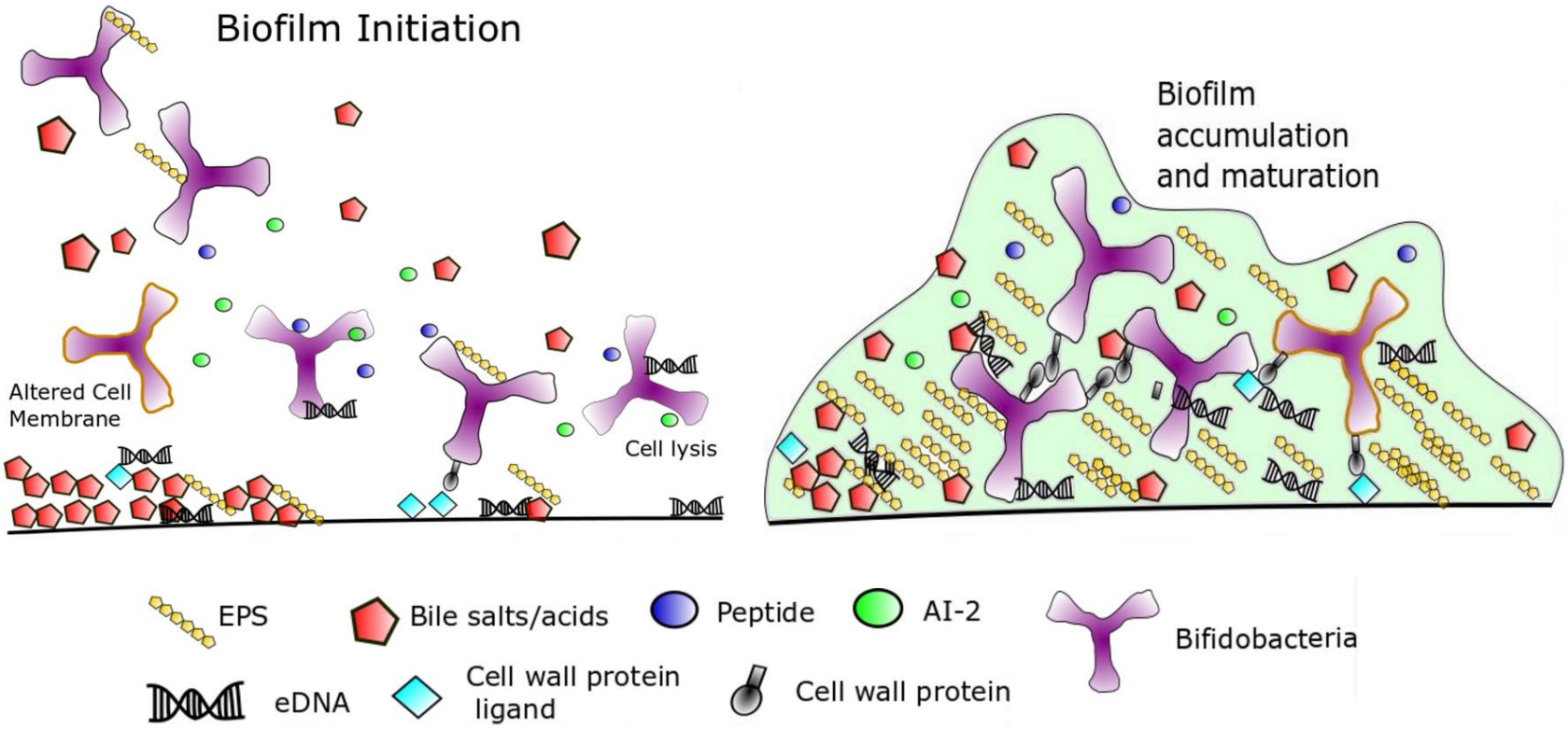
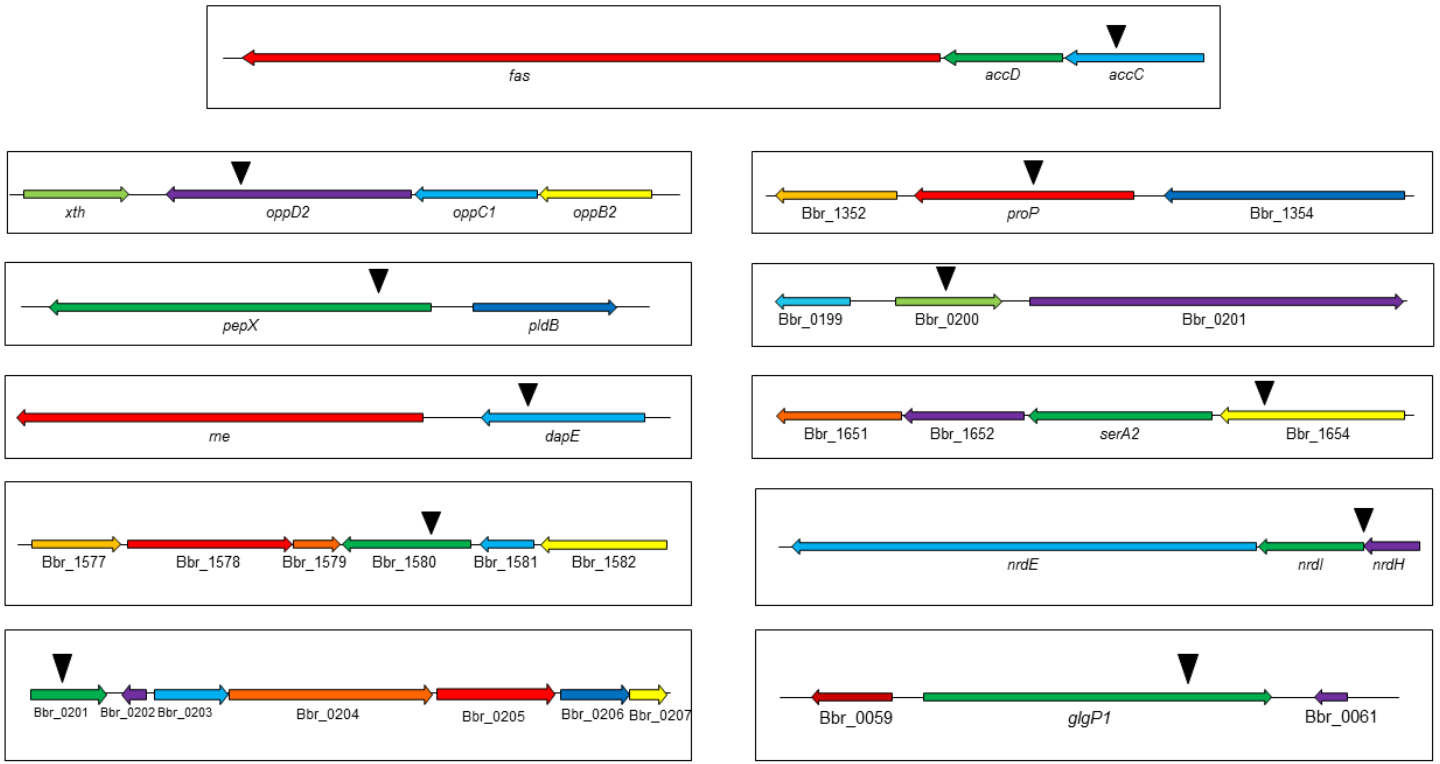


Figure 5.7. Model of biofilm formation by bifidobacteria induced by high concentrations of bile.

See discussion for details.



Supplementary Figure S5.1. Diagram of transposon insertions of biofilm mutants in *B. breve* UCC2003. Black triangles represent transposon insertions.

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Chapter VI

General Discussion and Future Perspectives

6.1 General discussion and future perspectives

Bifidobacteria are gut commensals that colonise infants at birth and continue to be part of the gut microbiota throughout the human lifespan, albeit in diminished abundance as the human host ages. Therefore, particular members of the *Bifidobacterium* genus, as representative autochthonous gut bacteria, have successfully adapted to the metabolic and physiological challenges of the GIT environment and are able to successfully colonise this habitat. Some of the environmental challenges that bifidobacteria face include low pH, bile acids/salts, nutrient acquisition, anti-microbial peptides and competition with other members of the gut microbiota. Indeed, some probiotic bacteria, which are considered allochthonous with respect to the human gut and which are supplied in certain commercial products, are believed to be incapable of GIT colonisation (1). This inability to colonize a human host may also be reflected in the genomes of certain bifidobacterial species; for instance, *Bifidobacterium animalis* subsp. *lactis* has undergone gene loss and genome decay and this may have happened as a result of long-term, continued cultivation growth under commercial production conditions (2). Therefore, investigations to discover and understand mechanisms by which bifidobacteria colonize and survive in the intestinal environment are warranted, and based on this premise particular aspects of carbohydrate metabolism and bile-induced biofilm formation were explored in this thesis.

Nutrient acquisition is vital for bifidobacterial survival in and colonisation of the gut. In the infant gut, breast milk provides HMOs which certain species/strains of bifidobacteria are adapted to consume either directly or indirectly through cross-feeding, and this is believed to be one of the main reasons why certain bifidobacterial species are highly prevalent and abundant in the infant gut. However, HMO levels reduce and eventually disappear as infants wean from breast milk and with the introduction of solid foods in the diet, and as a result the relative abundance of bifidobacteria in the gut substantially decreases. The relative abundance of members of the genus *Bifidobacterium* in the adult gut is reported to be between 4% and 6%, although this may vary among different populations. In order to survive bifidobacteria must be able to metabolise certain dietary carbohydrates to remain in the adult gut. In the adult diet such carbohydrates can be present in the form of fibre or plant-associated glycans derived from cereals amongst

other sources (3). Previous studies have shown that certain bifidobacteria are able to metabolize such plant-derived glycans, in particular the *B. longum* subsp. *longum* taxon (4, 5). However, plant-derived glycans are typically complex and sometimes insoluble polysaccharides, and metabolism of such carbohydrates is species/strain specific and may involve a range of enzymes encoded by multiple genetic loci. The scientific investigations described in this thesis were focused on plant-derived, arabinose-containing poly/oligosaccharide degradation by the *B. longum* subsp. *longum* taxon and included the functional characterisation of an esterase (Chapter II of this thesis) encoded by a gene located in a genomic locus presumed to be dedicated to arabino-oligosaccharide (AOS) metabolism (6). Furthermore, in Chapter III of this thesis three enzymes, presumed to represent an extracellular α-L-arabinofuranosidase, an extracellular arabinanase and an intracellular α-L-arabinofuranosidase, were assessed for their role in AOS metabolism. The obtained findings may explain certain carbohydrate utilisation differences with regards to arabinan and AOS metabolism between strains of the *B. longum* subsp. *longum* taxon. These findings also illustrate the complexity and apparent overlap between particular plant-oligo/polysaccharide metabolic pathways encoded by this taxon. Arabinan and AOS are therefore potential prebiotics for the *B. longum* subsp. *longum* taxon, although strain-specific metabolic abilities must be considered when developing arabinan/AOS-based prebiotic and synbiotics. Arabinan/AOS utilisation by bifidobacteria is believed to involve dedicated hydrolytic enzymes and transporters, which can be encoded by multiple distinct genetic loci. Therefore, the scientific findings of this thesis increased our knowledge regarding plant-derived poly/oligosaccharide metabolism, and specifically that of arabinan/AOS, in the *B. longum* subsp. *longum* taxon. Further studies should include the generation of isogenic mutants, phenotypic studies using a variety of different AOS substrates, and phenotypic complementation in order to determine the specific function of the genes involved in arabinan/AOS metabolism.

Furthermore, how dependent are bifidobacteria on other microbial species for cross-feeding these plant-oligosaccharides? It has previously been shown that bifidobacteria can cross-feed on arabinogalactan degraded by *Bacteroides* spp. (7, 8). More research is needed into the specific cross-feeding strategies that seem to exist between *Bacteroides* and bifidobacterial species. *Bacteroides* spp. are known for their

ability to degrade complex plant glycans (9), and they are called ‘messy eaters’ that extracellularly degrade glycans releasing oligosaccharides for other GIT members, including bifidobacteria, to scavenge (10). More detailed studies are needed to understand these complex ecological interactions, which may then allow rational strategies to be exploited for the development of novel plant-derived oligo/polysaccharide prebiotics. However, this also requires that plant-derived glycans are purified to a high quality, that the detailed structural (DP, covalent linkages and sidechain substitutions) information of these carbohydrates is known and that sufficient amounts of oligosaccharides are purified to allow growth and transcriptional analyses. Currently, plant oligosaccharides are not widely available in sufficient amounts and at a reasonable cost, while characterising oligosaccharides requires specialist techniques and expensive equipment such as mass-spectrometry, HPLC, HPAEC-PAD and NMR. Furthermore, following the acquisition of this information, animal models would need to be employed to assess the prebiotic/bifidogenic potential of a given oligosaccharide. Animal models may then also be employed to explore this research with arabinan/AOS as the main component of the supplied diet.

In the gut bifidobacteria are likely to have access to a variety of carbohydrates and it is important that they choose the most energy efficient carbon source to metabolise as bifidobacteria are competing with other microbial species in the gut. It is therefore crucial to understand the regulatory mechanism that enables bifidobacteria to preferentially choose their carbon source. Carbon catabolite repression (CCR) has been described for *B. longum* subsp. *longum* with an unusual preference for lactose over glucose (11). Interestingly, in *B. breve* a non-CCR system of global regulation has been reported with the ability to simultaneously regulate the uptake of several carbohydrate utilisation loci (12), although the precise details of this regulatory process have yet to be discovered. Therefore, there is still much to be understood about regulation of bifidobacterial carbohydrate metabolism at a global level. At a local level, LacI-type regulators are the predominant biological tool used for transcriptional regulation of a genetic locus involved in the utilization of a particular glycan. In Chapter IV we describe the LacI-type regulator AauR, which was shown to bind to a previously predicted operator binding sequence, although the effector of this presumed repressor could not be identified. Different plant-

oligosaccharides derived from hemi-celluloses and pectin have highly complex structures, yet in cases contain identical monomeric/oligomeric components and glycosidic linkages. Additionally, bifidobacterial genomes often contain multiple loci in different locations across the genome dedicated to the metabolism of dietary carbohydrates. It is likely that if bifidobacteria are provided with a buffet of plant-derived oligosaccharides to metabolise in the gut they must choose the most energetically favourable carbon source as they are competing for resources with other members of the microbiota. A better understanding of bifidobacterial transcriptional regulation of plant-derived oligosaccharides is needed to gain insights into preferential utilisation of prebiotic plant-derived carbohydrates.

In order to survive in the gut bifidobacteria, whether they are part of a probiotic product or when trying to colonize the infant gut, must be able to cope with exposure to bactericidal bile salts and bile acids (13). Bifidobacteria have developed various strategies to circumvent the antimicrobial activity of bile/bile salts including compositional changes to the cell wall (14), removal by multi-drug transporters (15), and changes in carbohydrate metabolism (16). Bile has been shown to induce biofilm formation in bifidobacteria (17) and biofilm formation is induced when LuxS is overexpressed (18). In Chapter V, we show that biofilm is induced by high concentrations of bile and also uncovered some of the molecular players involved in biofilm formation (19), thereby significantly advancing the current knowledge on bifidobacterial biofilm formation. Additionally, biofilm was shown to elicit a protective effect against the bactericidal properties of bile. This knowledge aids in the understanding of how bifidobacteria survive in the gut environment and biofilm is clearly an important lifestyle choice for colonisers of the GIT as bifidobacteria appear to form microbial biofilms on food debris in the gut (20). Furthermore, the EPS^- mutant that had reduced biofilm formation, as described in Chapter V, was previously shown to have a reduced colonisation persistence and increased sensitivity to 0.3% (w/v) porcine bile (21). This indicates that biofilm formation and the resulting potential protection from bile contributes to bifidobacterial gut colonisation. Further studies may focus on employing a murine model to determine if other mutants with reduced biofilm capacity are compromised in colonisation ability. Biofilm formation may be important to consider when selecting and delivering bifidobacterial strains as probiotic supplements; bifidobacterial strains with a greater capacity to form biofilm may

have an enhanced ability of reaching the large intestine alive. However, further knowledge about the molecular mechanisms that enable bifidobacterial gut colonisation is needed. In addition, it will be very interesting to assess if and how biofilm formation protects bifidobacteria against other stresses such as acid pH or anti-biotics, which may in turn enhance our knowledge on the mechanism by which bifidobacteria survive in the physiologically challenging gut environment.

The research described in this thesis has contributed to increased knowledge on bifidobacterial metabolism of arabinan/AOS, especially for members of the *B. longum* subsp. *longum* taxon and showed how complex and strain specific arabinan/AOS metabolism is within the *B. longum* subsp. *longum* taxon. This thesis showed that arabinan/AOS has a potential to be a prebiotic to stimulate the growth of strains in the *B. longum* subsp. *longum* taxon but also highlighted the need to understand the strain specific metabolism of bifidobacteria; therefore, it is important to choose the relevant strain and glycan/oligosaccharide in potentially products aimed at increasing bifidobacteria in the gut and enhancing their purported health benefits. This thesis also established that biofilm formation is an important and protective survival strategy for bifidobacteria in response to bile and is likely a key strategy for survival in the gut. Again, biofilm formation is another consideration in probiotic/synbiotic products that potentially aid probiotics survival in reaching the large intestine alive. Overall, this thesis increased our understanding of how bifidobacteria survive and persist in the gut environment which has implications when considering strains and prebiotics designed to increase bifidobacterial abundance in the gut and potentially bestowing their health benefits upon the host.

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