Discovery of proteins involved in the interaction between prebiotics carbohydrates and probiotics & whole proteome analysis of the probiotic strain Bifidobacterium animalis susp. lactis BB-12

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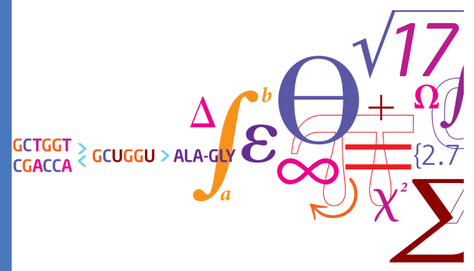

Ofir Gilad
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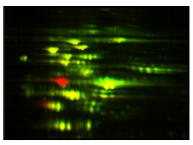

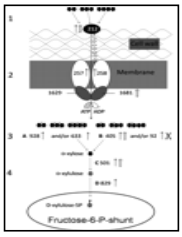

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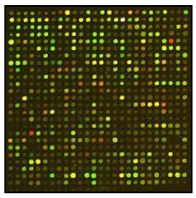

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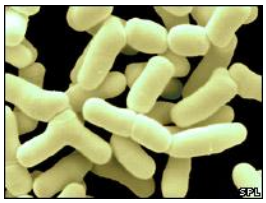

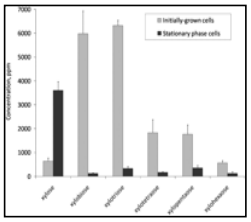

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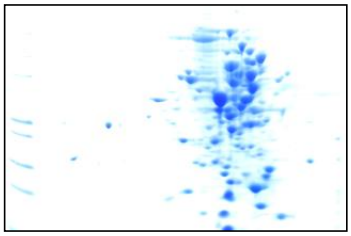

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Preface

This thesis represents the results of an industrial-PhD study carried out at the Enzyme and Protein Chemistry group (EPC), Department of Systems Biology, Technical University of Denmark (DTU) and at the Department for Identification, Chr. Hansen A/S for the period of March 2007 to July 2010. The project was partly founded by Danish ministry of Science, Technology and Innovation, the Danish Research Council for Natural Science and the Centre for Advanced Food Studies.

The present study has resulted in the following manuscripts:

Gilad O, Jacobsen S, Stuer-Lauridsen B, Pedersen MB, Garrigues C, Svensson B. (2010) Combined transcriptome and proteome analysis of *Bifidobacterium animalis* subsp. *lactis* BB-12 grown on xylo-oligosaccharides and a model of their utilization. Appl. Environ. Microbiol. 76:8275-8291 (Chapter 3).

Gilad O, Jacobsen S, Holm Viborg A, Stuer-Lauridsen B, Svensson B. Extracellular proteome analysis from *Bifidobacterium animalis* subsp. *lactis* BB-12 reveals proteins with putative roles in probiotic effects. Submitted for publication in Proteomics (Chapter 4).

Ofir Gilad, Karin Hjernø, Jacobsen S, Stuer-Lauridsen B, Svensson B, Jensen ON. Preliminary analysis of membrane proteins of *Bifidobacterium animalis* subsp. *lactis* BB-12. In Prep. (Chapter 5).

Holm Viborg A, Kim Ib Sørensen, Jacobsen S, **Gilad O**, Stuer-Lauridsen B, Svensson B. Recombinant production and chracterisation of a β-xylosidase/α-L-arabinofuranosidase from *Bifidobacterium animalis* subsp. *lactis* BB-12. In prep.

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Last but not least, I would like to give my special thanks to my family back home in Israel, for their long-distance support and encouragement during the project.

Summary

Probiotic bacteria, which primarily belong to the genera *Lactobascillus* and *Bifidobacterium,* are live microorganisms that have been related to a variety of health-promoting effects. Prebiotics are indigestible food components that specifically stimulate the growth of probiotic organisms in the human gastrointestinal tract. Despite an increased scientific focus within this field, the mechanisms behind the beneficial effects exerted by pre- and probiotics are still far from fully understood.

The purpose of the present industrial-PhD project was to identify proteins involved in interactions between the widely-used, extensively-studied probiotic strain *Bifidobacterium animalis* subsp. *lactis* BB-12 and potentially-prebiotic carbohydrates. The project was initiated with a screening phase in which more than 40 carbohydrates were tested for their ability to promote the growth of the bacterium. The results showed that BB-12 can utilise a wide range of oligosaccharides like galacto-, iso-malto malto-, soybean-, xylo- and possibly also fructo-oligosaccharides.

Xylo-oligosaccharides (XOS) have been selected for further studies with respect to the proteins that play a role in their catabolism in BB-12. These studies included comparative transcriptome and proteome analysis of samples obtained from BB-12 cultures grown on XOS (with glucose as a reference). These analyses indicated that the expression of a putative gene cluster with relevance for XOS catabolism is increased during growth on XOS. Based on these results and on chromatographic analysis of XOS consumption in BB-12 cultures, a model for XOS utilisation in BB-12 was established.

The subsequent phase of the PhD project included extracellular proteome analysis, giving rise to the identification of 86 unique proteins. Of these proteins, 33 are potentially related to interactions between the bacterium and host intestinal cells. These interactions consist of adhesion, recruitment of human plasminogen and immunomodulation. In addition, proteins related to nutrient uptake and cell wall turnover were also found to be secreted by the bacterium.

The final part of the project included the identification of 250 proteins (86 of them contain transmembrane segments) from the bacterial membrane fractions, including 61 proteins associated with transport systems with various predicted substrates such as oligosaccharides, amino acids and inorganic ions. In addition, 7 of the 8 subunits of the H^+-ATPase enzyme complex and the majority of the proteins that embody the translocation machinery were identified.

The results obtained in the present study may have an impact at both the scientific and the industrial levels. In addition to knowledge acquired regarding XOS catabolism in BB-12 and the implications on the design of a synbiotic preparation based on the two, the proteins identified in this study that are predicted to be involved in molecular mechanisms underlying probiotic effects or other essential physiological processes can serve as promising targets for detailed investigations, whose results may be of great relevance from both the applicable and scientific perspective.

Dansk Resumé

Probiotiske bakterier, som primært tilhører taksonerene *Lactobacillus* og *Bifidobacterium*, er levende mikroorganismer som ved indtagelse medfører sundhedsfremmende virkninger. Præbiotika er ufordøjelige fødevarekomponenter, som specifikt stimulerer væksten af probiotiske organismer i tarmen. På trods af et øget videnskabeligt fokus på området er mekanismerne bag disse funktionelle fødevarekomponenters gavnlige effekter stadig langt fra fuldt forstået. Formålet med dette erhvervs-PhD projekt var at kortlægge de proteiner, der er involveret i samspillet mellem den bredt-anvendte og velstuderede probiotiske stamme *Bifidobacterium animalis* subsp. *lactis* BB-12 og potentielt-præbiotiske kulhydrater. Projektet blev indledet med en screeningsfase, hvor mere end 40 kulhydrater blev testet for deres evne til at fremme væksten af BB-12. Resultaterne viste, at bakterien kan omsætte en bred vifte af oligosakkarider som galacto-, iso-malto-, malto-, soybønner-, xylo- og måske også fructo-oligosakkarider. Xylo-oligosakkarider (XOS) er blevet valgt for videre studier af proteinerne, som spiller en rolle i oligosakkaridernes omsætning i BB-12. Studierne omfattede komparativ transkriptom- og proteomanalyse af prøver fremstillet af BB-12 kulturer dyrket på XOS (med glukose som reference). Analyseresultaterne viste, at genekspression af et formodet gencluster med relevans for XOS omsætning er forøget under vækst på XOS. Baseret på disse studier samt kromatografisk analyse af XOS omsætning, er der blevet etableret en model for XOS-katabolisme i BB-12. Den efterfølgende del af projektet havde fokus på de proteiner som bakterien secernerer til omgivelserne. Denne ekstracellulære proteomanalyse har ført til identificering af mange proteiner med potentielle funktioner i samspillet mellem BB-12 og værtens tarmceller. Projektets sidste del omfattede identificering af 250 proteiner (86 af dem indeholder transmembran-doegmenter) fra bakteriens membranfraktion. Disse bestod af transportører for f.eks. oligosakkarider, aminosyrer og uorganiske ioner. Resultaterne opnåede i dette projekt kan have en betydning på både den videnskabelige og den anvendelsesmæssige plan. Den opnåede viden omkring XOS katabolismen kan anvendes i en eventuelt synbiotisk præparation baseret på XOS og BB-12. Ydermere, kan mange af de identificerede proteiner, som potentielt er involveret i de molekylære mekanismer bag probiotiske effekter og andre essentielle fysiologiske processer, bruges som subjekter for målrettede studier, der kan have stor relevans fra både det anvendelsesmæssige og det videnskabelige perspektiv.

"רֵאשִׁית חָכְמָה קְנֵה חָכְמָה וּבְכָל-קִנְיָנְךָ קְנֵה בִינָה". (משלי ד';7)

"Wisdom is the principal thing; therefore get wisdom: and with all thy getting get understanding". (Proverbs 4; 7)

Table of contents

List of Abbreviations

ABC	ATP-binding cassette
ACN	Acetonitrile
ANOVA	Analysis of variance
AXOS	Arabinoxylan-oligosaccharides
CFU	Colony-forming unit
CoA	Coenzyme A
Cys-HCl	Cysteine-hydrochloride
Da	Dalton
EPS	Exopolysaccharides
ESI	Electrospray ionisation
FA	Formic acid
FOS	Fructo-oligosaccharides
F6P	D-Fructose-6-phosphate
GEO	Gene expression omnibus
GIT	Gastro-intestinal tract
GOS	Galacto-oligosaccharides
HMB	Human breast milk
IBD	Inflammatory bowel diseases
IEF	Isoelectric focusing
IMO	Isomalto-oligosaccharides
LC	Liquid chromatography
LGG	*Lactobacillus rhamnosus GG*
MFS	Major facilitator superfamily
MOS	Malto-oligosaccharides
MP	Membrane proteins
MNOS	Manno-oligosaccharides
MS	Mass spectrometry
Mw	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National centre biological information
NDOs	non-digestible oligosaccharides
NFW	Nueclease-free water
ORF	Origin of replication
PCR	Plolymerase chain reaction
PDB	Protein data bank
PHGG	Partially-hydrolysed guar gum
pI	Isoelectric point
PMT	Photo Multiplier tube
PTS	Phosphotransferase system
pNP-	*para*-nitrophenyl-
qTOF	Quadrupole time-of-flight
RP	Ribosomal proteins
SBP	Solute-binding proteins
SCFA	Short-chain fatty acids
SDS	Sodium dodecyl sulfate
SOS	Soybean oligosaccharides
TFA	Trifluoroacetic acid
TIGR	The institute for genomic research
Tris	Trishydroxymethylaminomethane
XOS	Xylo-oligosaccharides
UDP	Uridine diphosphate
X5P	D-Xylulose-5-phosphate
2-DE	Two-dimensional gel electrophoresis

CHAPS	3-[3-Cholamidopropyldimethylammonio]-1-propanesulfonate
HPAEC-PAD	High performance anion exchange chromatography with pulsed amperometric detection
MALDI-TOF	Matrix-assisted laser desorption/ionization-time-of-flight
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SILAC	Stable isotope labelling of amino acids in cell culture
2D-DIGE	Two-dimensional differential gel electrophoresis

Chapter 1. Introduction

1.1. Probiotic bacteria

The human gastrointestinal tract (GIT) is harbored (or transiently colonised) by more than a 1,000 phylotypes belonging to 8 primary phyla (Rajilić-Stojanovic *et al*. 2007), which represent at least 800 genera and 16,000 species, with genera like *Bacteriodes*, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Fusobacterium* and *Ruminococcus* being most abundant. This dense microflora exhibits a larger genetic diversity compared with the human genome (Gil *et al*. 2006). In addition, these microorganisms provide the host with highly-valuable physiological traits such as utilisation of complex carbohydrates, vitamin biosynthesis, regulation of fat storage (Candela *et al*. 2010) and modulation of the host immune system (Sansonetti and Medzhitov 2009). Moreover, the intestinal microbiota serves as a barrier against colonisation of host epithelial cells by pathogenic microorganisms and induces the regeneration and barrier functions of the epithelium (Neish 2009). The gut microflora acquires nutrients from two major sources – endogenous mucus glycans secreted by host epithelial cells and plant-derived complex carbohydrates that escape digestion in the upper GIT.

During the past couple of decades, the awareness to the quality and the nutritional content of food has markedly increased. This enhanced interest has been accompanied with dedication of growing attention and resources to the field of functional food, which aims at introducing health-promoting ingredients as supplements to daily nutrition. Two of the pivotal elements within this area are probiotic and prebiotics (prebiotics are described in more details in Section 1.2). Probiotic microorganism are defines as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host." (FAO/WHO 2002). In spite of the fact that probiotics have been consumed in fermented food products like yogurt, sausages and pickles for thousands of years, the attention of the scientific community started arising only in the beginning of the 20'th century. The majority of probiotic microogranims belong to the genera *Lactobacillus* and *Bifidobactreium*, but a few strains were also documented in other genera such as *Streptococcus, Ruminococcus, Enterococcus, Lactococcus, Leuconostoc*, *Pediococcus, Saccharomyces* and a single strain from *Escherichia* (Shah *et al.* 2007; Ewaschuk and Dieleman 2006). The numerous health-promoting effects ascribed to probiotics include prevention and treatment of GIT infections and inflammatory bowel diseases (IBD), immunomodulation, which is related both to prevention of allergy-associated diseases and prevention and treatment of GIT infections and inflammatory bowel diseases (IBD), immunomodulation, which is related both to prevention of allergy-associated diseases and

stimulation of the immune system through the use of adjutants in vaccination (Borchers *et al.* 2009), treatment of genitourinary infections (e.g. bacterial vaginosis; Krauss-Silva *et al.* 2010) and prevention of dental caries (He *et al.* 2009). Recent studies suggest that probiotics may also contribute to combating liver diseases like low-grade inflammation, hepatic fat infiltration and hepatitis (Gratz *et al.* 2010), and to reduction in the levels of carcinogens in rats (Gratz *et al.* 2006) and biomarkers associated with liver cancer risk (El-Nezami *et al.* 2006).

The health-benefiting effects introduced by probiotic bacteria are derived therefore either from their direct interactions with host cells and proteins in the lower GIT, or indirectly, via inhibition of the growth of pathogenic bacteria. Such inhibition mechanisms consist of production of short-chain fatty acids (SCFA) that lower the pH and thus attenuate the growth of some harmful intestinal strains, competitive adhesion to host intestinal epithelial cells or tissues like collagen and laminin, and production of antimicrobial toxins, also known as bacteriocins (Gillor *et al*. 2008). In addition to that, since the bifidobacterial species identified so far lack the enzymes urease, azo- and nitro-reductase, β-glucuronidase and α-dehydrolase, whose action result in introduction of toxic conounds (Sánchez et *al*. 2010).

The principal rationale underlying the administration of probiotics, prebiotics, and the combination of the two – synbiotics, is to alter the human (or animal) GIT microbiota in favor of probiotic strains on the expense of pathogenic microorganisms like certain strains of the species *Clostridium difficile, Clostridium perfringens*, *Salmonella enterica, Salmonella flexneri, Campylobacter jejuni*, *Bacteroides fragilis*, *Escherichia coli, Helicobacter pylori*, *Vibrio cholerae* and some *Bacillus* species (Schauder and Bassler 2001).

1.1.1. Bifidobacteria

Bifidobacteria are non-motile, non-sporulating, non-gas-producing, rod-shaped, facultative anaerobic, saccharolytic, high G+C Gram-positive bacteria belonging to the Actinobacteria class and the *Bifidobacteriaceae* family (Ventura *et al.* 2007c). Bifidobacteria have been isolated from five different ecological niches: the intestine, the oral cavity, fermented food, the insect gut, and sewage (Ventura *et al.* 2007c). Of the mucosa-adherent bacteria in the intestine, bifidobacteria are among the most abundant commensal groups, accounting to nearly 10% of the microbiota in adults. Noticeably, the bifidobacterial population seems to be significantly different amongst different individuals (Turroni *et al.* 2008). As late as January 2009, 30 species were identified from the genus *Bifidobacterium*, which are divided to 6 phylogenetic clusters: *B. longum, B.*

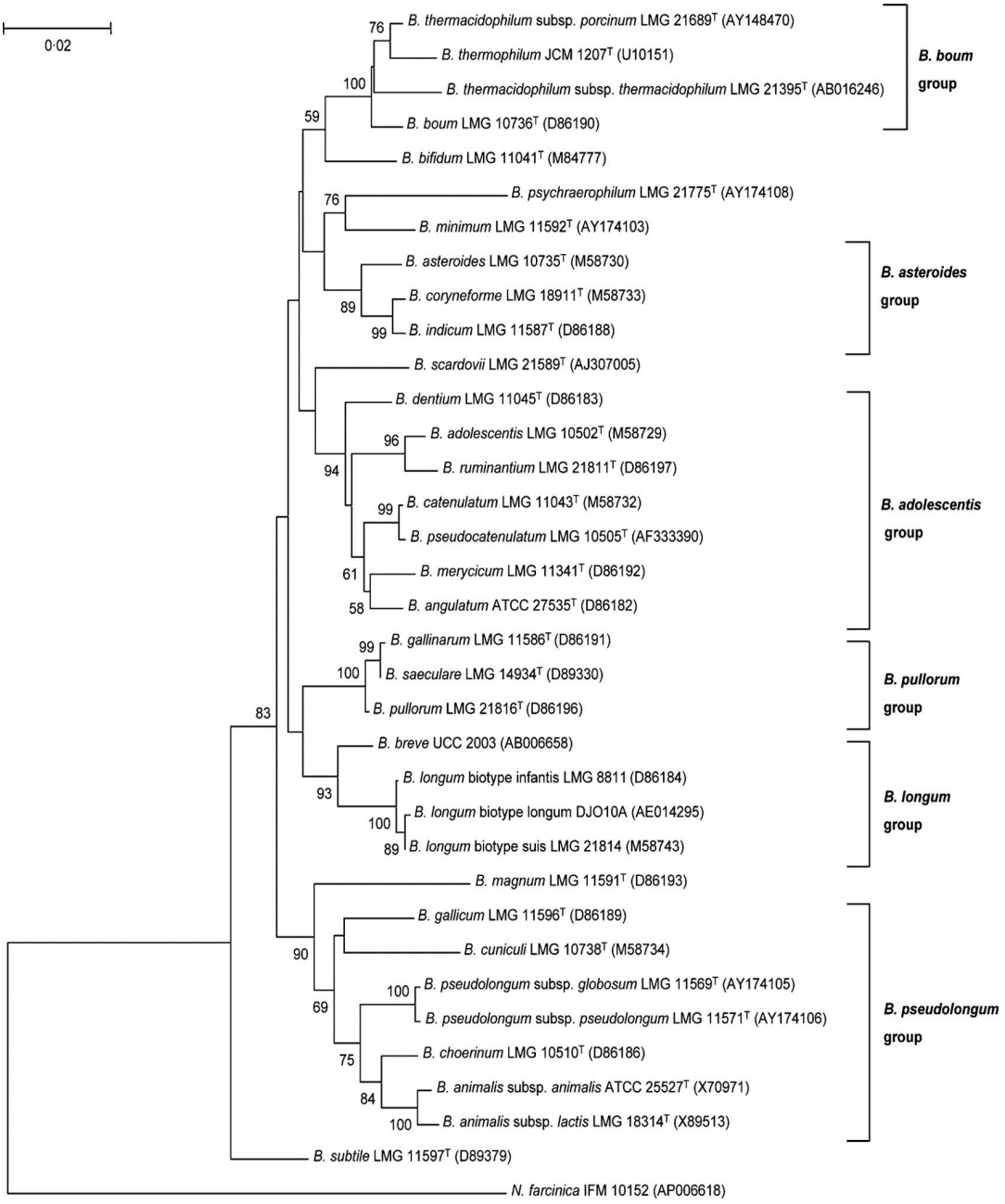


Figure 1.1. Phylogenetic tree of the genus *Bifidobacterium* based on 16S rRNA gene sequences. The tree was rooted using *Nocardia farcinica* IFM 10152. The bar designates 0.02 substitutions per nucleotide position. Adapted from Ventura *et al*. (2006).

pseudolongum, B. adolescentis, B. boum, *B. asteroides* and *B. pullorum*, (Fig. 1.1; Ventura *et al.* 2007c). The genomes of bifidobacteria are 1.9 to 2.9 Mb in size and resemble typical bacterial chromosomes with respect to general architectural features such as co-orientation of gene transcription and DNA replication (McLean *et al.* 1998) and a presumptive origin of replication region (ORF) (Qin *et al* 1999).

Currently, the genome sequences of over 20 strains from 10 different species (e.g. *B. longum, B. bifidum, B. adolescentis, B. animalis, B. breve*, *B. dentium*, *B. angulatum*, *B. pseudocatenulatum*, *B. catenulatum* and *B.* gallicum) have been made publically available. Comparison of the identified strains led to the observation that the genus *Bifidobacterium* exhibits a low level of phylogenetic and genomic diversity (Ventura *et al.* 2006), yet a few exceptions from this syteny, where inversions, deletion or insertion of DNA regions uniquely found in a single genome have been identified. The majority of these genetic elements, which were probably incorporated into bifidobacterial genomes by horisontal gene transfer, consist of prophage-like elements, restriction modification systems, integrative plasmids and genes associated with exopolysaccharides (EPS) biosynthesis, as well as genes related to phage defense. Whole genome comparison also illustrated that the adaptation of bifidobacteria to the human GIT is not exclusivly related to their developed fermentative capabilities for complex carbohydrates, but also to their ability to obtain essential nutrients like vitamins, amino acids and nucleotides (Ventura *et al*. 2007b).

As described above, numerous bifidobacterial strains have been documented to be associated with probiotic effects. Consequently, the number of studies concerning these effects, as well as investigations of the physiology (as seen by the genomic and proteomic features) of probiotic stains of the genus *Bifidobacterium* has been remarkably increasing during the past two decades. Searching the term **"***Bifidobacterium"* in NCBI's Pubmed http://www.ncbi.nlm.nih.gov/pubmed?term=Bifidobacterium, for instance, gives currently (10 June 2011) rise to 3751 hits to publications related to members of this genus.

The catabolic enzymatic arsenal of bifidobacterial strains isolated from neonates seems to be oriented towards utilisation of milk oligosaccharides, whereas in strains prevalent in adults the majority of the sugar-degrading enzymes have plant oligosaccharides as substrates (Sela *et al*. 2010). These complex carbohydrates, some of which regarded as prebiotics, can be administrated in combination with probiotic cultures or as an independent preparation, with the aim to elevate the levels of bifidobacteria (along with other probiotic strains) in the intestine. The utilisation of these carbon sources is facilitated primarily through their degradation by a wide battery of glycoside hydrolases that cover a broad range of potential substrates like galacto-, fructo-, iso-

malto-, malto- and xylo-oligosaccharides (GOS, FOS, IMO, MOS and XOS, respectively), as well as other hydrolases active against starch. In addition to glycosidases, the breakdown of poly- and oligosaccharides also involves the action of glycosyl transferases, which transfer sugar moieties from a donor molecule to a sugar acceptor. In the case of insoluble polysaccharidic substrates, carbohydrate-binding modules (CBMs; Boraston *et al.* 2004), which are distinct amino acid sequence within a carbohydrate-active enzyme that has a discreet protein fold with carbohydrate-binding activity, can facilitate binding to these substrates (van den Broek *et al*. 2008a).

In addition to their versatile catabolic arsenal, bifidobacteria also possess a plentitude of transport system, mostly for di- and oligosaccharides. The majority of the sugar transport systems in bifidobacteria are of the ATP-binding cassette (ABC)-type (see section 3.2 for a more detailed description), and to a lesser extent the phosphoenol pyruvate-dependent phosphotransferase system (PTS), in which transport is accompanied by concomitant phosphorylation. Another type of transporters found in bifidobacteria are those of the major facilitator superfamily (MFS), which are ubiquitous single-polypeptide secondary carriers capable exclusively of translocating small solutes in response to chemiosmotic ion gradients (Pao *et al*. 1998).

Bifidobacteria are predicted to utilise two primary strategies for uptake of sugars, both associated with the combined action of the carbohydrate-acting enzymes and transporters. The principal difference between the two strategies is related to whether carbohydrate degradation takes place intracellularly or extracellularly. According to the first mechanism, poly- and oligosaccharides are bound at the cell surface (potentially via CMBs), cleaved by membrane- or cell wall-bound hydrolases, and the resulting monosaccharides and short-chain oligosaccharides (depending on the mechanism of action of the enzyme) are subsequently transported across the membrane. Such a strategy was described for arbino-xylan catabolism in *B. longum* biotype *longum* (van den Broek *et al*. 2008a).

According to the second strategy, at the absence of extracellular hydrolases or transferases, degradation of carbohydrates takes place intracellularly as a following step to sugar transport, as was suggested for GOS utilisation in *B. animalis* subsp. *lactis* DR10 (Gopal *et al.* 2001). A similar model for XOS catabolism is described in Section 3.4.

The monosaccharides resulting from carbohydrate degradation are then incorporated into the fructose-6-phosphate shunt, the central carbohydrate metabolic pathway characteristic for bifidobacteria, also known as the bifid shunt (Fig. 1.2). The key enzyme of this pathway is fructose-6-phosphate phosphoketolase, which catalyzes the conversion of D-fructose-6-phosphate (F6P) into D-erythrose-4-phosphate and acetyl phosphate, and the primary end products of the

pathway are lactate and acetate. The bifid shunt produces three moles of acetate, two moles of lactate and five moles ATP per two moles utilised hexose. However, studies have shown that the theoretical ratio of 1.5 mole acetate/lactate may depend on the abundance of the carbon source and its consumption rate, such that catabolism is diverted in favor of generation of formate and acetate when the carbon source levels become scarce, as was reported for *B. animalis* IPLA 4549 (Ruas-Madiedo *et al.* 2005) and *B. animalis* subsp. *lactis* BB-12 (Van der Meulen *et al*. 2006), probably since this shift increases the ATP yield of the shunt.

1.1.1.1. *Bifidobacterium animalis* subsp. *lactis* BB-12

B. animalis subsp. *lactis* BB-12 is an extensively used commercial probiotic strain isolated from fermented milk and cultured in 1983 by Chr. Hansen A/S. The strain is included in a variety of food applications and dietary supplements, and is ascribed various probiotic effects such as treatment of allergy, prevention and treatment of diarrhea, improvement of oral vaccination, reducing cholesterol levels *in vitro* (Alhaj *et al*. 2010) and several immunomodulative effects (Schiffrin *et al*. 1995; Isolauri *et al*. 2000; Mitsuma *et al*. 2008; Ouwehand *et al*. 2008). In addition, combined probiotic preparations of BB-12 and *Lactobacillus acidophilus* NCFM showed enhanced bowel movement frequency (Alm *et al*. 1993).

As a strain used in orally-administered probiotic preparations or as a food additive, BB-12 possesses two pivotal physiological traits that promote its ability to survive the transition through the highly acidic gastric juice (pH of approx. 2.0 [Campbell 1996]) in the stomach and the high bile salt levels in the intestine. Among five bifidobacterial strains tested for their acid tolerance, BB-12 was the only strain whose survival rates were not significantly altered upon exposure to high acid concentrations (pH 2; Vernazza *et al*. 2006). The acid resistance of BB-12 is thought to be related to the action of a F_0F_1-ATPase complex (Sánchez *et al.* 2007), which is a pH-inducible proton pump facilitating the export of hydrogen ions (see also Chapter 5).

Bile salts are cholesterol-derived detergent-like compounds synthesised in the liver and stored as amino acid conjugates in the gall bladder. Subsequent to digestion, they are secreted into the intestine where they assist in emulsification and absorption of lipids. As a result of their amphiphilic nature**,** bile salts are also potent antimicrobial agents through the damaging of bacterial membranes, and were also described to impose oxidative stress on bacterial DNA (Bernstein *et al*. 1999). The two primary enzymes involved in bile salt tolerance in BB-12 are choloylglycine hydrolase (also known as bile salt hydrolase; EC 3.5.1.24) that hydrolyses six major human bile salts (Kim *et al*. 2004) and the oxalate-degrading oxalyl coenzyme A decarboxylase (EC 4.1.1.8; Federici *et al*. 2004). Additional possible mechanisms for bile salt

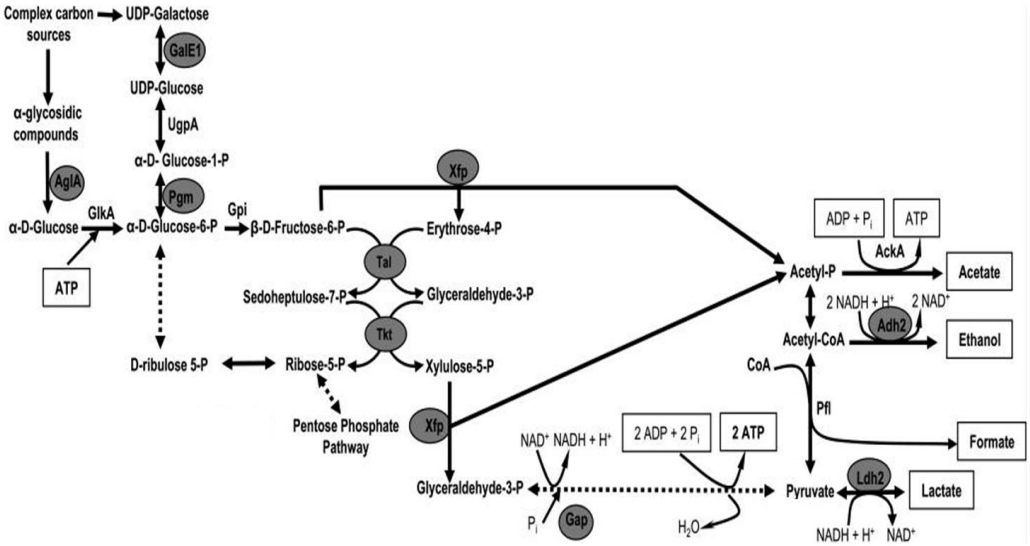


Figure 1.2. **Schematic illustration of the fructose-6-P shunt (bifid shunt). Abbreviations: AckA, acetate kinase; Adh2, aldehyde-alcohol dehydrogenase 2; Aga, α-galactosidase; Agl, α-glucosidase; GalE1, UDP-glucose 4-epimerase; Gap, glyceraldehyde-3-phosphate dehydrogenase C; GlkA, glucokinase; Gnt, 6-phosphogluconate dehydrogenase; Gpi, glucose 6-phosphate isomerase; Ldh2, lactate dehydrogenase; Pgm, phosphogluco-mutase; Pfl, formate acetyltransferase; Tal, transaldolase; Tkt, transketolase; UgpA, UTP-glucose-1-phosphate uridylyltransferase; Xfp, xylulose-5-phosphate/fructose-6-phosphate phosphoketolase; Zwf2, glucose-6-phosphate 1-dehydrogenase; Pi, phosphate. Adapted from Sánchez *et al*. (2007).**

tolerance, as suggested for *B. animalis* subsp. *lactis* IPLA 4549, include: (i) induction of the bifid shunt and its diversion towards the acetic branch to increase ATP yield; (ii) enhancement of the degradation of oxalate with the subsequent formation of formate; (iii) up-regulation of redox active proteins like methionine synthase, ketol-acid reductoisomerase and *O*acetyl-homoserine sulfhydrolase; (iv) over-production of general stress response proteins like the chaperones DnaK, ClpB, GrpE, GroES and GroEL; (v) induction of proteins related to DNA repair (e.g. carbamoylphosphate synthase); and (vi) alteration of lipid metabolism, which may also have an impact on the lipid composition of cell membranes (Sánchez *et al*. 2007).

Another important feature that is pivotal in the context of the present study is the ability of BB-12 to ferment a variety of oligosaccharides, as documented by *in vitro* growth experiments (Chapter 2). These phenotypic findings are in accordance with the genomic content of the bacterium, whose genome contains a large number of genes coding for a variety of potential oligosaccharidic-degrading enzymes. The capability of BB-12 to ferment a variety of carbon sources is described in detail in Chapters 2–3.

1.2. Prebiotic carbohydrates

A prebiotic substrate was initially defined as a "nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health." (Gibson and Roberfroid 1995). As the scientific and industrial interest rose with the years, many plant poly- and oligosaccharides were claimed to possess prebiotic properties. To obtain an unambiguous definition of the term that could remove doubts regarding whether a given component can be regarded as prebiotic, Roberfroid formulated a more stringent definition, according to which "a prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confer benefits upon host well-being and health" (Roberfroid 2007). In addition, Roberfroid also added three criteria to the definition: (i) resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption; (ii) fermentation by intestinal microflora; and (iii) selective stimulation of the growth and/or activity of those intestinal bacteria that contribute to health and well-being. According to these new definition and criteria, only two types of prebiotic candidates accomplish the requirements – oligufructans (inulin and FOS) and GOS (Roberfroid 2007), yet data concerning other carbohydrates like XOS, IMO, lactosucrose, gluco- and soybean oligosaccharides (SOS), as well as polydextrose, are pointing towards the addition of a few candidates (regarded as “emerging prebiotics”) to the list, when the results of clinical studies will be sufficiently solid. The investigation of other potential prebiotic components like gluconic acid, resistant starch, melibiose-, mannan- pectin- and *N*-acetylchitin-derived oligosaccharides, lactose-, glutamine- and hemicellulose-rich substrates, as well as germinated barley foodstuffs, oligodextrans and lactoferrin-derived peptides may also give rise to the discovery of novel prebioitic sources (Gibson *et al*. 2004).

1.2.1. Prebiotics substrates for Bifidobacteria

Since *Bifidobacterium* is one of the two major probiotic genera, they have been extensively investigated with respect to screening for potential prebiotic candidates with bifidogenic properties. The majority of these studies have been carried out using *in vitro* growth experiments with single bifidobacterial strains, while recent, more complex experiments involve fecal inocula and or the use of a colon simulator (Mäkeläinen *et al*. 2009a,b). A representative list of studies documenting bifidogenic substrates is designated in Table 1.1. As can be seen from the table (and mentioned in Section 1.1.1.), bifidobacteria are capable of fermenting a wide range of poly- and especially oligosaccharides. Some studies have shown that the catabolising specificities of bifidobacteria is not only species-specific, but also strain-specific, as reported in a study by

Vernazza and co-workers (2006), where only one of the two *B. longum* strains tested grew on FOS and the specific growth rate of two strains varied considerably upon growth on XOS, IMO, lactulose, and a mixture of FOS and inulin. The fermentative capabilities of BB-12 are described in detail in Chapters 2 and 3.

It should be stressed, however, that the bifidogenic carbohydrates listed in Table 1.1 do not necessarily meet all of the three requirements to be classified as prebiotics (formulated by Roberfroid), as they also have to withstand digestion and hydrolysis in conditions mimicking the human upper GIT, and meet an even more challenging requirement related to specific stimulation of growth of probiotic microorganism without induction of growth of pathogenic microorganisms. In this context it should be clarified that usage of the term "prebiotic(s) in this report is of practical reasons, and does not necessarily mean that the respective carbon source meet the aforementioned criteria to be regarded as prebiotics.

1.3. Genomics and proteomics in bifidobacteria

1.3.1. Transcriptomics-based screening for prebiotics-active proteins

As a consequence of the continuously increasing interest in the field of pre- and probiotics, extensive resources have been invested in broadening the knowledge related to their mode of action. The vast advancements that have been simultaneously taking place in genomic and proteomic technologies provide researchers with highly-valuable bioinformatics tools utilised to study genes/proteins that seem to play a role in a given molecular mechanism.

The first substantial progress in bifidobacterial genomics took place when Schell and colleagues (2002) published the genome sequence of *B. longum* NCC2705, the first *Bifidobacterium* strain whose genome sequence became publicly accessible. Unraveling the genomic content of this important human GIT-derived strain demonstrated that it seems to have adapted to its biological niche, in the form of an extensive repertoire – both in variety and quantity – of enzymes capable of degrading complex carbohydrates. Analysis of the genomes of NCC2705 and *B. adolescentis* ATCC 15703 showed that 8% of their genomic content is dedicated to carbohydrate metabolism, which is comparable with that found in *Bacteroides,* another commensal genus isolated from the GIT, but 30% higher than non-GIT bacteria like *Lactococcus lactis* and other intestinal microorganisms as *Escherichia coli* or *Enterococcus faecium* (Ventura *et al*. 2007c). Further investigation of the transport systems of NCC2705 lead to the identification of 19 putative transport systems for mono-, di- and oligosaccaharides, 13 of which belonging to the ABC-type transport systems (Parche *et al*. 2007). These transport systems are clustered in distinct operon organisation consisting of sugar-binding and permease proteins,

glycoside hydrolases, and in some cases a regulator gene located adjacent to the target operon.

Along with sequence-based homology searches against known transport proteins, Parche *et al*. also performed comparative cDNA microarrays analysis of selected genes (with glucose as a reference carbon source) predicted to belong to the above-mentioned transport systems. The results showed that the all of the genes coding for proteins related to carbohydrate transport were expressed, and some of these genes were up-regulated on some of the carbon sources tested (lactose, maltose, raffinose and FOS; genes up-regulated on raffinose and FOS are listed in Table 1.2). The fact that a few of these genes were induced by both lactose and FOS suggests that the respective transport system may be promiscuous.

The effect of growth on human milk-derived oligosaccharides of the human infant strain *B. longum* LMG 13197 on the physiology of the strain and mechanisms associated with probiotic effects were investigated. Samples obtained from cultures grown on human breast milk (HMB), milk formula and GOS were subjected to microarray hybridisation assay, with glucose as a reference (González *et al*. 2008). A few putative genes related to degradation of mucin and human milk oligosaccharides, as well as to *N*-acetylglucosamine degradation, were up-regulated upon growth on HBM compared to milk formula. The expression patterns obtained in the GOS samples were similar to those of the HMB samples, giving rise to increased expression of genes predicted to encode cell surface type 2 glycoprotein-binding fimbriae that may be involved in attachment and colonization in the intestine. Similar proteins were identified from extracellular protein samples of BB-12 (Section 4.3.3.3.2.). The similar expression patterns observed in the HMB and the GOS cultures may indicate that the bifidogenic effect in human milk is related to its rich oligosaccharide content. The results of this study were supplemented by transcriptional profiling based on a mixed species microarray analysis applied to bifidobacterial RNA isolated from rapidly processed fecal samples from neonates fed with HBM or a formula containing a mixture of GOS and FOS (Klaassens *et al*. 2009). In accordance with the study by González *et al*. (2008), the majority of the transcripts identified were associated with carbohydrate metabolism. In addition, 18 genes related to degradation of plant- or mucin-derived oligosaccharides were identified, including genes coding for enzymes with potential substrates as starch, GOS, XOS, arabino-oligosaccharides, lacto-*N*-biose, fucose, and *N*-acetylgalactosamine (Klaassens *et al*. 2009). The successful application of the mixed-bifidobacteria microarrays approach shows that unsequenced bifidobacterial strains can also be analysed using this methodology, as long as a complete genome sequence of a related strain is available and hence enables mapping of the intestinal transcriptome of bifidobacteria.

Table 1.1. Selected carbohydrates supporting the growth of bifidobacteria.

Prebiotic candidate	Methodology	Strain	Reference
Inulin	*in vitro*; single strain	- *B. breve* 15698 - *B. infantis* 17930 - *B. adolescentis* 15706 - *B. longum* 15708	Huebner *et al.* 2007
		- *B. longum* KN29.1 - *B. catenulatum* KD14 - *B. animalis* KD12	Bielecka *et al*. 2002
		- *B. animalis* subsp. *lactis* BB-12	Vernazza *et al*. 2006
FOS	*in vitro*; single strain	- *B. breve* 15698 - *B. infantis* 17930 - *B. adolescentis* 15706 - *B. longum* 15708 - *B. bifidum* NCI	Huebner *et al.* 2007
		- *B. adolescentis* DSM20083 - *B. animalis* subsp. *lactis* BB-12 - *B. longum* 46	Vernazza *et al*. 2006
	in vitro; fecal inucula	- *B. pseudolongum* KSI9 - *B. bifidum* KD6 - *B. animalis* KD10 - *B. catenulatum* - *B. globosum* - *B. angulatum*	Bielecka *et al*. 2002
GOS	*in vitro*; *single strain*	- *B. breve* 15698 - *B. infantis* 17930 - *B. adolescentis* 15706 - *B. longum* 15708	Huebner *et al.* 2007
		- *B. bifidum* CECT 870	Pérez-Conesa *et al*. 2005
		- *B. adolescentis* DSM20083 - *B. infantis* DSM20088 - *B. animalis* subsp. *lactis* BB-12 - *B. longum* 46	Vernazza *et al*. 2006
Lactulose	*in vitro*; *single strain*	- *B. longum*. H5-2	Tu *et al*. 2008
		- *B. adolescentis* DSM20083 - *B. infantis* DSM20088 - *B. animalis* subsp. *lactis* BB-12 - *B. longum* 46	Vernazza *et al*. 2006
IMO	*in* vitro; single strain	- *B. adolescentis* DSM20083 - *B. infantis* DSM20088 - *B. animalis* subsp. *lactis* BB-12 - *B. longum* 46	Vernazza *et al*. 2006
IMO	*in* vitro; single strain	- *B. adolescentis* DSM20083 - *B. infantis* DSM20088 - *B. animalis* subsp. *lactis* BB-12 - *B. longum* 46	Vernazza *et al*. 2006
	in vitro*;* fecal inucula	- *Bifidobacteria*[¶]	Rycroft *et al*. 2001
Polydextrose	*in vitro;* single strain	- *B. adolescentis* DSM20083 - *B. infantis* DSM20088 - *B. longum* 46	Vernazza *et al*. 2006
Polydextrose	clinical studies; fecal microflora enumeration	- Bifidobacteria	Jie *et al*. 2000
	in vitro; fecal inucula	- Bifidobacteria	Rycroft *et al*. 2001
Pectin, Pectic-oligosaccahrides	*in vitro*; fecal inucula	- Bifidobacteria	Olano-Martin *et al*. 2002
SOS	*in vitro*; fecal inucula	- Bifidobacteria	Rycroft *et al*. 2001

Raffinose	*in vitro*; single strain	- *B. adolescentis* MB 239	Amaretti *et al.* 2006
		- *B. longum* H10-6	Tu *et al*. 2008
		- *B. animalis subsp. lactis* BB-12 - *B. longum* 2 - *B. infantis* 420	Jaskari *et al*. 1998
XOS	*in vitro*; single strain	- *B. infantis* Bb-02 - *B. adolescentis* VTT E-981074 - *B. angulatum* ATCC 27535 - *B. bifidum* Bb-11 - *B. breve* VTT E-81075 - *B. catenulatum* ATCC 27539 - *B. gallicum* ATCC 49850 - *B. infantis* VTT E-97796 - *B. animalis subps. lactis* BB-12 - *B. longum* VTT E-96664 - *B. pseudocatenulatum* ATCC 27919 - *B. pseudolongum* 25526	Crittenden *et al*. 2002
	colon simulator; fecal inocula	- *B. animalis* subsp. *lactis* group‡ - Bifidobacteria	Mäkeläinen *et al*. 2009a
Arabinoxylan	clinical studies; fecal microflora enumeration	- *B. adolescentis* VTT E-991436 - *B. longum* VTT E-96664	Jie *et al*. 2000
AXOS (arabinoxylan oligosaccharides)	*in vitro*; single strain	- *B. adolescentis* ATCC 15703	Van Laere *et al*. 2000
Arabinogalacto-oligosaccharides		- *B. adolescentis* ATCC 15703 - *B. longum* ATCC 15707 - *B. breve* ATCC 15700 - *B. infantis* ATCC 15697	
Arabino-oligosaccharides		- *B. longum* ATCC 15707	
Panose	colon simulator; fecal inocula	- Bifidobacteria - *B. animalis* subsp. *lactis* group	Mäkeläinen *et al*. 2009b
Resistant starch	*in vitro*; single strain	- *B. adolescentis* ATCC 15703 - *B. angulatum* ATCC 27535 - *B. breve* ATCC 15699 - *B. longum* Lafti B22	Crittenden *et al.* 2001
Starch, amylopectin, pullulan	*in vitro*; single strain	- *B. breve* NCFB 2258 - *B. dentium* NCFB 2243 - *B. infantis* CCUG 45868 - *B. pseudolongum* NCIMB 2244 - *B. thermophilum* JCM 7027	Ryan *et al*. 2006

¶ Quantitative-PCR based counts for strains of the bifidobacteria group.
‡ Quantitative-PCR based counts for strains of the *B. animalis* subsp. *lactis* group.

Until recently, genetic studies of bifidobacteria at the molecular level have been relatively limited, as a result of hurdles introduced by some of their physiological characteristics (e.g. strict anaerobicity, nutritional fastidity and instability of DNA cloning in *E. coli*). In parallel with the increasing knowledge within both novel molecular techniques and the sequencing of an increasing numbers of bifidobacterial strains, cloning vectors based on a cryptic plasmid from *Bifidobacterium catenulatum* L48 have been developed (Alvarez-Martín *et al*. 2008). Such vectors may be utilised for the design of plasmids used delivery of antigens and tumor-

Table 1.2. **Bifidobacterial genes coding for carbohydrate-active proteins up-regulated on different carbon sources (compared to glucose) according to cDNA microarrays analysis.**

Strain	Carbon source	Predicted substrate for operon	Encoded protein	Locus tag	Reference
***B. longum* NCC2705**	FOS	Ribose	Sugar ABC transporter solute-binding protein	BL0033	Parche *et al*. 2007
			ABC transporter permease	BL0035	
			Sugar ABC transporter permease	BL0036	
		Maltose	Arabinosidase	BL0146	
		lactose, FOS	Solute binding protein of ABC transporter system	BL0262	
		FOS	Solute binding protein of ABC transporter system	BL0425	
		lactose, FOS	Sugar ABC transporter solute-binding protein	BL1164	
			Sugar ABC transporter solute-binding protein	BL1165	
		FOS	Solute binding protein of ABC transporter for sugars	BL1638	
			Permease of ABC transporter for sugars	BL1639	
			Permease of ABC transporter for sugars	BL1640	
		multiple sugar, mannos, fructose	Sugar binding protein of ABC transporter for pentoses	BL1694	
			ABC transporter permease	BL1696	
		sucrose	β-fructofuranosidase	BL0105	
			Galactoside permease	BL0106	
			LacI-type transcriptional regulator	BL0107	
		Lactose	LacZ	BL0978	
	raffinose	Raffinose	Sugar binding protein of ABC transporter system	BL1521	
			Sugar permease of ABC transporter system	BL1522	
			Sugar permease of ABC transporter system	BL1523	
		multiple sugar, mannose, fructose	ABC transporter permease	BL1696	
		Lactose	LacZ (β-Galactosidase)	BL0978	
***B. longum* LMG 13197**	human breast milk; GOS	FOS	Solute binding protein of ABC transporter for sugars	BL1638	González et al. 2008
			Permease of ABC transporter for sugars	BL1639	
		Unknown	Glycosyltransferase	BL1674	
		Unknown	LacZ (β-Galactosidase)	BL0978	
		N-acetyl-glucos-amine	N-Acetylglucosamine-6-phosphate deacetylase	BL1344	
			Glucosamine-6-phosphate deaminase	BL1343	
		lactose, FOS	Sugar ABC transporter solute-binding protein	BL1164	
			Sugar ABC transporter solute-binding protein	BL1165	
		Unknown	Glycosyltransferase	BL0672	
		Lactose	Galactoside symporter	BL0976	
		Unknown	Probable α-1,4-glucosidase	BL0529	
		Unknown	α-L-arabinosidase	BL0544	
		lactose, FOS	Sugar ABC transporter permease	BL1169	
***B.adolescentis* ATCC 15703**	human breast milk	Unknown	Pullulanase	BAD_0708	Klaassens *et al*. 2009
		Unknown	Probable sugar kinase	BAD_1412	
		Unknown	Endo-1,4-β-xylanase	BAD_1527	
		Unknown	β-Galactosidase	BAD_1605	
B. longum NCC2705		(A)XOS, FOS	Possible arabinosidase	BL0146	
		unknown	Narrowly conserved HP possibly involved in xylan degradation	BL0421	
		unknown	Probable-1,4-glucosidase; maltase-like enzyme	BL0529	
		unknown	Sucrose phosphorylase	BL0536	
		unknown	α-L-Arabinosidase	BL0544	

***B. lonum* NCC2705**		unknown	Glycogen phosphorylase	BL0597	Klaassens *et al*. 2009
		unknown	Glycosyl hydrolase (LacZ)	BL0978	
		unknown	Possible glycosyltransferase	BL1104	
		mucin sugars	Solute-binding protein of ABC transporter for sugars	BL1638	
			Permease of ABC transporter for sugars	BL1639	
			LNB phosphorylase	BL1641	
			Galactose-1-phosphate uridylyltransferase	BL1643	
			UDP-glucose 4-epimerase	BL1644	

¶ Genes identified by microarrays hybridization human breast milk; no comparative expression was considered in this context.

suppressing substances (Rossi *et al*. 1996; Fujimori 2006) and enhancing beneficial detoxifying activities (Park *et al*. 2007). Efficient systems for introduction of the gene of interest into these expression systems have, however, yet to be further developed.

1.3.2. Proteomics in bifidobacteria

To complement findings obtained at the transcriptional level, a proteomic analysis is commonly applied. Studies at the proteomic level can both corroborate results from transcriptional studies and yield results not detected at the gene level, thereby contributing to a more comprehensive understanding of the role bifidobacterial proteins play in probiotic effects or physiological related to colonisation of the host GIT and survival in this environment.

Despite of its limitations, e.g. bias against protein with low abundance, proteins with extreme mass and iso-electric points and hydrophobic proteins like membrane proteins, two-dimensional gel electrophoresis (2-DE) is a central approach for deciphering concrete physiological conditions in the cell, by providing a snapshot of the protein expression pattern at the analysed condition (Beranova-Giorgianni 2003; Fountoulaki *et al*. 2004; Conrotto and Souchelnytskyi 2008). Proteomics is also useful for studying the amount and subcellular location of individual proteins and for identification of genes that are affected by regulatory proteins.

The application of proteomic techniques for the analysis of the protein content of bifidobacteria has advanced in parallel with improvements related to the marked increase in information derived from databases. Thishas been facilitated by both the growing number of bifidobacterial genomes sequence being made available, and the introduction of highly sensitive mass spectrometers. These advances, especially with regard to instrumentation, made it feasible to identify hundreds (in some cases thousands) of proteins from a biological sample using a high-throughput approach. A few studies regarding implementaion of proteomic-based methodologies are described below and listed in Table 1.3.

The first application of proteome analysis in general and a high-throughput method in particular in bifidobacteria was illustrated in a study carried out by Vitali and coworkers (2005)

regarding proteome analysis of *Bifidobacterium infantis*. The authors utilised the so-called multidimensional protein identification technology (Mudpit; Link *et al*. 1999), according to which proteins are separated by strong cation exchange (SCX) chromatography implemented as a primary separation technique, followed by reversed-phase chromatography concomitant with electrospray ionisation (ESI) tandem mass spectrometry. Applying this shotgun approach, 136 proteins were identified, including proteins with high mass and basic isoelectric point (pI) that are commonly underrepresented in gel-based proteomic studies. The majority of the proteins identified in this study, which are considered to be the most abundant under exponential growth of the bacterium, were related to cell survival, replication and energy metabolism. The production of proteins associated with stress defense mechanisms in the absence of any stress stimuli implicate that bifidobacteria constitutively produce these proteins, probably to attain rapid cellular response.

A metaproteomic approach involving 2-DE and matrix-assisted laser desorption/ ionization-time of flight mass spectrometry (MALDI-TOF MS) was utilised to identify proteins isolated from infant fecal microbiota (Klaassens *et al*. 2007). The analysis showed that the proteomic content of the fecal micobiota changed over the course of time, and peptides obtained from tryptic digestion of the proteins extracted from the spots on the gels showed sequence similarity to bacterial proteins, a viral protein, and four eukaryal proteins, but none of these peptides gave rise to significant database hit, which can be explained by the fact that the majority of the microbiota has not been cultured yet. A single peptide sequence detected, however, was homologous to bifidobacterial transaldolase, thus providing experimental evidence to the presence of bifidobacterial strains in the human neonate intestine.

To broaden the knowledge into the mechanisms of bile salt tolerance in bifidobacteria, 2-DE followed by MALDI-TOF MS was applied on protein samples from *B. longum* NCIMB 8809 and *B. animalis* subsp. *lactis* IPLA 4549 and its bile tolerance deficient mutant 4549dOx grown in the presence of bile or without it. These studies gave rise to the identification of 37 and 34 differentially-abundant proteins for 8809 and IPLA 4549, respectively. As mentioned in section 1.1.1.1., these analyses showed that the strains induce several complementing physiological mechanisms upon exposure to bile (Sánchez *et al*. 2005; Sánchez *et al*. 2007).

Another investigation of bile tolerance in bifidobacteria, which incorporated more advanced methodologies, was performed by Ruiz *et al*. (2009). This study utilised differential fractionation and digestion of bacterial cell wall and membrane fractions followed by two-dimensional differential gel electrophoresis (2D-DIGE) and stable isotope labelling of amino acids in cell culture (SILAC) to map the cell envelope proteome of the above-mentioned 8809 strain grown in

Table 1.3. Selected studies applying proteomic approaches in bifidobacteria.

Feature analysed	Bifidobacterial strain(s)	Analysis method	Reference
Shotgun protein identification	*B. infantis* BI07	MudPIT	Vitali *et al*. 2005
Effects of bile on protein expression patterns	*B. longum* NCIMB 8809	2-DE → MALDI-TOF MS	Sánchez *et a*l. 2005
Effects of bile on protein expression patterns	*B. animalis* subsp. *lactis IPLA 4549 & 4549dOx*	2-DE → MALDI-TOF MS	Sánchez *et al*. 2007
Identification of extracellular proteins	*B. longum* NCIMB 8809	2-DE → MALDI-TOF MS	Sánchez *et al*. 2007
Metaproteomic analysis of infant fecal microbiota		2-DE → MALDI-TOF MS	Klaassens *et al*. 2007
Proteome analysis of cell envelope under bile exposure	*B. longum* NCIMB 8809	2D-DIGE →LC-LTQ MS & SILAC → MALDI-TOF MS	Ruiz *et al*. 2009
Identification of regulated proteins in co-culture compared to pure cultures	Co-culture of *B. longum* NCIMB 8809 and *B. breve* NCIMB8807	2-DE → MALDI-TOF or LC-ESI MS	Ruiz *et al*. 2009
Proteome analysis & comparison of samples from glucose and fructose-grown cultures	*B. longum* NCC2705	2-DE → MALDI-TOF MS	Yuan *et al*. 2006

an *in vitro* bile environment. 2D-DIGE, which was also applied in the present study (Chapter 3), is based on the same electrophorating principals as 2-DE, and utilises the sensitive protein-binding properties of flourophoric cyanin dyes which enable the analysis of two biological samples and an internal standard in a single gel run (Marouga *et al*. 2005). The SILAC approach applies radiolabeling-based quantitative analysis of samples, an approach that may be highly valuable for the analysis of membrane proteins whose resolving in traditional gel-based methods is difficult (Ong *et al*. 2002). The results of this study are described in detail in Section 5.2.

Another example for the application of 2-DE supplemented with MALDI-TOF MS was illustrated by an analysis of the effect of co-culturing of *B. longum* NCIMB8809 and *B. breve* NCIMB8807. Comparison of the protein content of cytosolic extracts from early stationary phase cells showed that the abundance of 16 proteins was altered when the strains were co-cultures compared to growth in pure cultures. These included 10 ribosomal proteins (7 of which up-regulated), a transcriptional regulator involved in regulation of stress-related proteins, a phosphoketolase and a UDP-*N*-acetylglucosamine: LPS N-acetylglucosamine transferase (MurG), a glycosyltransfe-rase essential to peptidoglycan biosynthesis and cell division. The activity of the latter against *para*-nitrophenyl (*p*NP)-*N*-acetyl-β-D-glucosamine was increased in *B. longum* NCIMB8809 grown in the presence of *B. breve* NCIMB8807.

An analysis of extracellular proteins in bifidobacteria was applied on culture supernatants of *B. longum* NCIMB 8809 (Sánchez *et al*. 2008b). Seven-teen protein spots detected on the 2-D gel yielded identification of 10 unique proteins, including solute-binding proteins, proteins homologous to bacterial conjugation proteins, a protein with sequence similarity to an invasion-associated protein and putative enzymes involved in peptidoglycan metabolism.

The application of 2-DE coupled with MALDI-TOF and liquid chromatography (LC)-ESI MS was also applied in the comprehensive proteome analysis of *B. longum* NCC2705, which in addition conferred comparison of the protein abundance patterns of cultures grown on fructose and glucose (Yuan *et al*. 2006). This study resulted in the identification of 369 proteins associated with a variety of cellular pathways that account for 30% of the predicted proteome. It was also reported that although fructose and glucose are utilised *via* the same degradation pathway, fructose uptake may be mediated by a specific transport system whose two of its components showed increased abundance on fructose compared to glucose. Another example for the utilisation of comparative proteomics for inference of sugar catabolism is described in detail in Chapter 3, whereas identification of extracellular and membrane proteins in BB-12 is discussed in Chapters 4 and 5, respectively.

1.4. The objectives of the study

Probiotic bacteria are incorporated in food or food supplements due to their well documented health benefits, especially with respect to the human GIT. Prebiotics are food components that specifically stimulate the intestinal growth of probiotic microorganisms. The attention given to prebiotics, probiotics and synbiotics is continuously increasing from both the industrial and academic point of view. With respect to carbohydrates considered as prebiotic candidates, only GOS and fructans (FOS and inulin) meet the criteria required to be regarded as prebiotics, yet a handful of plant-derived oligosaccharides seem to have promising potential to be added to this list.

As a first step for evaluating possible prebiotic candidates able to support the growth of the widely studied and extensively used probiotic strain *B. animalis* subsp. *lactis* BB-12, a few dozens of carbon sources were tested by *in vitro* growth experiments. The results of these studies are described in Chapter 2.

Another aspect of the interaction between pre- and probiotics concerns the catabolic mechanisms that facilitate the utilisation of prebiotic carbohydrates by probiotic bacteria. A wide battery of glycoside hydrolases has been characterised from bifidobacteria (van den Broek *et al*. 2008b), and it is therefore expected that BB-12 also possesses similar enzymes that play a role in

degradation of oligo- and polysaccharidic substrates. The objective of the next step in the present study was thus to identify proteins involved in the catabolism of the selected carbon sources, with the aim to pinpoint candidates for further characterisation. Such genes/proteins predicted were identified by comparative transcriptomic and proteomic analyses of samples grown on the selected substrates (with glucose as the reference), and corroborated by qPCR. The combination of the results from this analyses and biochemical characterisation of the corresponding proteins was utilised for establishment of a model for the catabolism of these carbon sources by BB-12, as discussed in Chapter 3.

Another central aspect within the field of probiotics is the mechanisms underlying their health-promoting effects, which are, still far from fully deciphered. The objective of Chapter 4, which deals with the secreted proteome of BB-12, is thus to shed more light into the proteins that may take part in the interactions with the host GIT, as well as the identification of extracellular proteins with other physiological roles (e.g. nutrient uptake and cell wall metabolism).

Finally, Chapter 5 focuses on the membrane proteome of BB-12. As membrane proteins play pivotal role in a variety of cellular mechanisms like transport, signal transduction and communication with the exterior of the cell, mapping the membrane proteome is expected to broaden the knowledge regarding other physiological processes taking place in the bacterium.

Chapter 2. Screening for prebiotic candidates supporting the growth of *Bifidobacterium animalis* subsp. *lactis* BB-12

2.1. Summary

As the first step of the present study regarding prebiotics-related proteins of the probiotic strain *Bifidobacterium animalis* subsp. *lactis* BB-12, a variety of carbohydrates were tested for their performance in supporting the growth of the bacterium *in vitro*, yielding a detailed list of possible prebiotic candidates containing different oligosaccharidic species (e.g. iso-malto-, malto-, galacto-, transgalacto-, xylo- and soy-oligosaccharides). The experimental results were compared with the predicted catabolic capabilities of BB-12 as inferred from its genome sequence, as well as with studies regarding the utilisation of complex carbohydrates other bifidobacteria.

2.2. Introduction

Bifidobacteria isolated from the human lower gastrointestinal tract (GIT) seem to have gone through a genome reduction causing a shift in their metabolic capabilities from biosynthesis to metabolism of an extensive number of carbon and nitrogen sources (Barrangou *et al*. 2009). Complex carbohydrates that are not digested by the degradative enzymes of the host are the most abundant nutrients in the lower GIT, the ecological niche of bifidobacteria. The adaptation of bifidobacteria to this milieu is reflected by a wide spectrum of glycoside hydrolases capable of degrading these substrates (van den Broek *et al*. 2008b). Comparison of the variety and quantity of three publicly available bifidobacterial genome sequences shows that although *B. animalis* subsp. *lactis* Bl-04 has a lower number of glycoside hydrolases and also a smaller genome, the diversity of glycoside hydrolases in this strain is similar to that found in *B. adolescentis* ATCC 15703 and *B. longum* subsp. *longum* NCC2705 (Barrangou *et al*. 2009). However, the predicted substrate specificities of the glycoside hydrolases present in these strains are different, giving rise to diverse catabolic capabilities.

The environmental adaptation of bifidobacteria is also illustrated by the extent and variety of transport systems utilised in uptake of oligosaccharides, as exemplified by analysis of the genome sequence of *B. longum* subsp. *longum* NCC2705 (Schell *et al*. 2002; Parche *et al*. 2006). Oligosaccharide transport in this strain is predicted to be facilitated by 15 transport systems, the majority of which are ABC-type transporters capable of importing di- and oligosaccharides, and to a lesser extent by MFS or PTS transporters. An ABC transport system commonly consists of a sugar-binding protein attached to the cell membrane facing towards the surrounding medium, a couple of permease proteins creating a pore in the membrane, and a

couple of ATP-binding proteins that supply the energy required for the action of the transport system through ATP hydrolysis (Davidson *et al*. 2008). A handful of bifidobacteria have been reported to obtain higher cell yields and higher specific growth rates when cultivated on oligosaccharides compared to the respective monosaccharides (van der Meulen *et al*. 2004). These observations may be explained by efficient transport systems specific for di- and oligosaccharides.

A few of the aforementioned glycosidic enzymes and transport systems have been studied throughout the last decade. Proteins from species like *B. adolescentis, B. animalis, B. bifidum, B. breve, B. longum* and *B. pseudolongum* have been identified and cloned. These proteins were recombinantly produced (commonly in *E. Coli*) and subjected to molecular characterisation (Van den Broek *et al*. 2008b).

In spite of the growing knowledge of the metabolic features of different bifidobacterial species with regards to complex carbohydrate catabolism, the insight into the mechanisms by which bifidobacteria in general, and BB-12 in particular, utilise these substrates, is somewhat limited. In the present study, a variety of carbohydrates were tested for their ability to support the growth of BB-12. The *in vitro* approach was supplemented by mining the genome sequence of BB-12 for genes coding for proteins that may play a role in prebiotics catabolism (some of which clustered together in putative operons), as well as an overview of the current knowledge of the prebiotics-related catabolic capabilities of closely related bifidobacterial strains. The results of this study served as the basis for choosing substrates that were further analysed with respect to the proteins predicted to play a role in the catabolism of the chosen carbohydrates. The identification of these proteins will, in turn, broaden the insight into the mechanisms behind probiotics-prebiotics interactions in BB-12.

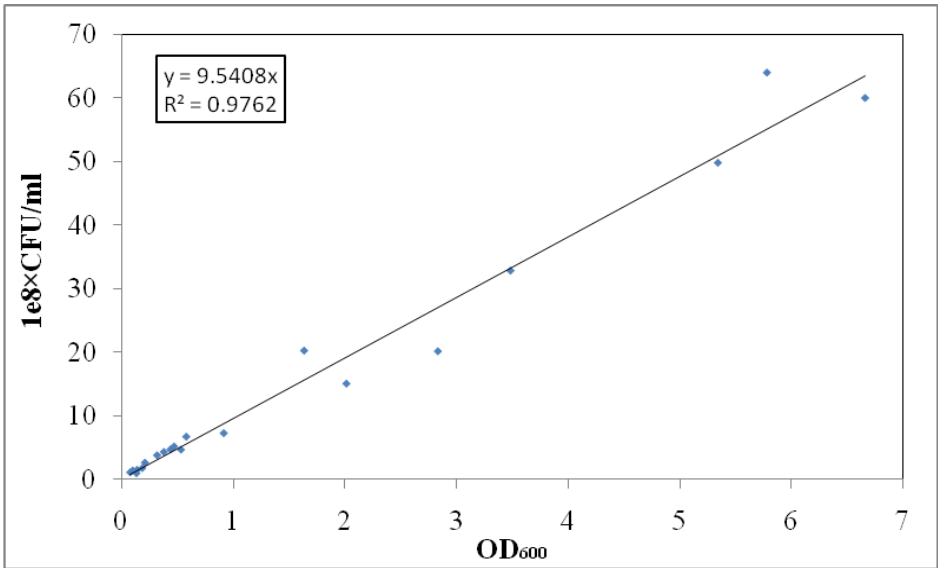
2.3. Results

2.3.1. Correlation between CFU/ml and OD_{600}

In order to rule out the possibility that some of the turbidity in the tested cultures is a result of non-viable cells, a dataset of 20 data points (after discarding of outliers; taken from cultures grown on different carbon sources and different cell morphologies, as was observed by microscopical studies) was established. A few outliers that deviated significantly from the general trend, were excluded from the correlations. For each measured OD value a corresponding viable count value was obtained by performing a dilution series and plating out the diluted cultures. These data point pairs were then used to plot the concentration of the viable cells in CFU$\times 10^8$/ml as a function of OD_{600} (Fig. 2.1), and a linear correlation (taking all 20 data points into account)

of $1\times OD = 9.5\times10^8$ CFU/ml was obtained, with $R^2 = 0.9762$. This correlation is very similar to the value obtained (for several bifidobacterial strains) by Pérez-Consa *et al.* (2005), according to which $1\times OD_{600} = 9.8\times10^8$ CFU/ml.

Figure 2.1. Correlation between CFU/ml and OD_{600} in BB-12 grown on a variety of carbon sources. The linear trend line, the obtained linear correlation and the correlation factor (R^2) are designated.



2.3.2. Growth experiments

The carbohydrates (2% w/v) were tested for their performance in supporting the growth of BB-12 cultures grown on a rich medium. The growth was monitored as OD_{600} readings along a 24 h period, or based on the ratio between the viable counts after 24 h of incubation and prior to inoculation.

2.3.2.1. Triplicate growth experiments of selected carbohydrates

The performance of selected prebiotic candidates was testes by three biological replicates of the 24 h-long growth experiment. The OD values measures for each time point in the three experiments were averaged, and these values, as well as the standard deviation (represented by vertical error bars) are illustrated in the following graph (Fig. 2.2):

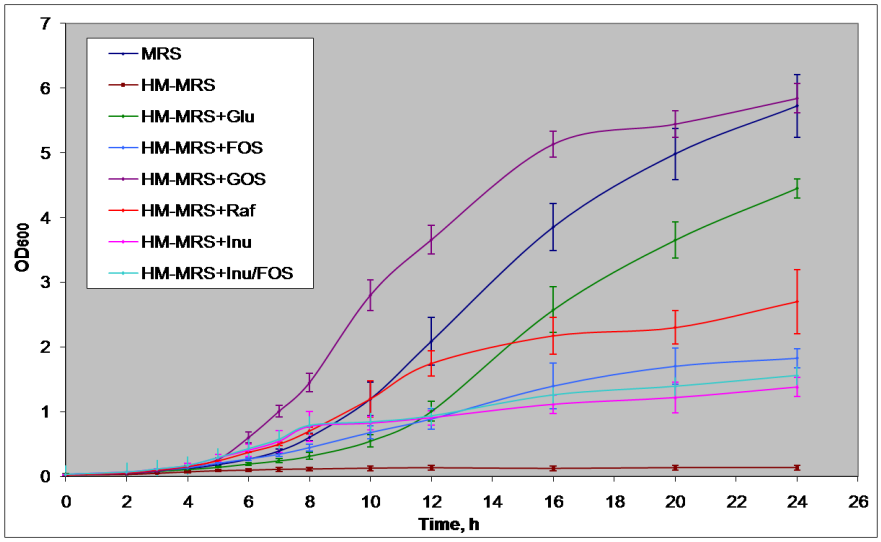


Figure 2.2. **Growth experiments of BB-12; the carbon sources correspond to the following numberes in the carbohydrate table (Table 2.2); Glu, glucose, reference carbon source; FOS, fructo-oligosaccharides, #1; GOS, galacto-oligosaccharides, #23; Raf, raffinose, #28; Inu, fructose enriched inulin, #3; Inu/FOS, 1:1 w/w mixture of inulin and FOS, #4; HM-MRS, MRS-like reconstituted medium with no carbon-source.**

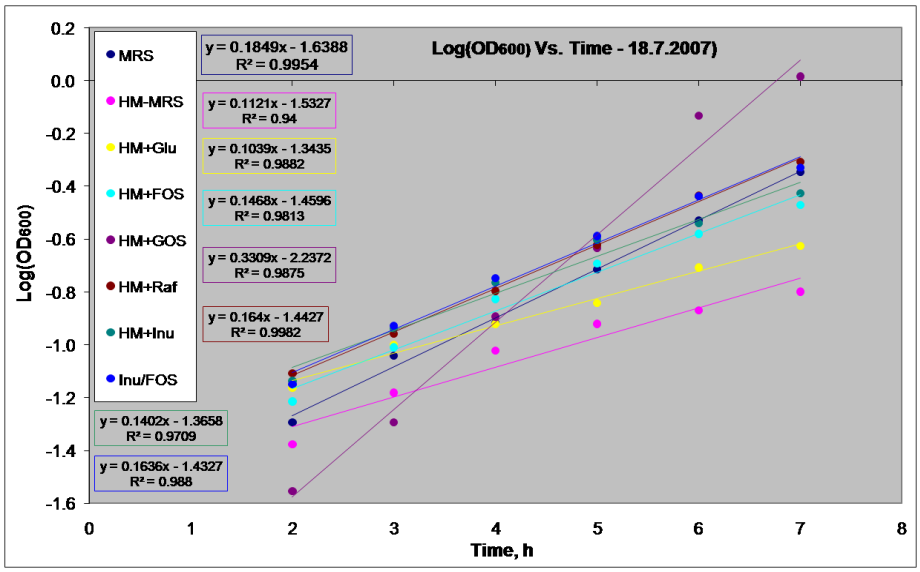


Figure 2.3. **Exponenetial growth phase of BB-12 cultures grown on a variety of carbon sources, as well as positive (MRS and HM-MRS+glucose) and negative (HM-MRS) controls. The logarithm of the OD_{600} values of growth curves is plotted as a function of time (a representative set of growth curves is shown). The linear trend line obtained and the R^2 (correlation factor) are designated. HM-MRS, home-made MRS; Glu, glucose; Raf, raffinose; Inu, inulin.**

Kinetic Calculations

The results of the growth rate calculations are based on the OD values obtained in the triplicate growth experiments described above, and are summarised in Figures 2.3–2.4 and Table 2.1.

Table 2.1. The doubling times and specific growth rates (and the standard deviation thereof) of the selected media/carbon sources.

Medium/C-source	Doubling time, min	Growth rate, 10×(1/h)
MRS	102 ± 6.1	5.9 ± 0.34
HM-MRS	225 ± 7.6**	2.9 ± 0.89
HM-MRS+Glucose	148 ± 25	4.1 ± 0.71
HM-MRS+FOS	112 ± 14	5.4 ± 0.74
HM-MRS+GOS	69 ± 16	8.9 ± 2.0
HM-MRS+Raffinose	100 ± 8.9	6.0 ± 0.52
HM-MRS+Inulin	110 ± 16	5.9 ± 0.34
HM-MRS+Inulin/FOS	99 ± 13	6.2 ± 0.87

** The growth curve of HM-MRS culture did not give rise to an exponential growth profile, such that the linear correlation of the curve was relatively poor. It is never the less included in the calculation as sugar-free control.

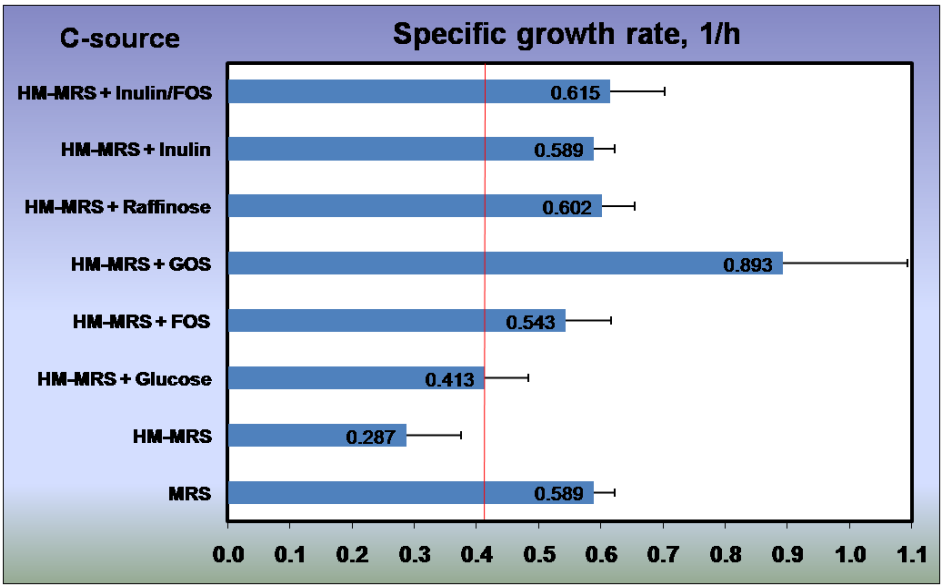


Figure 2.4. Specific growth rates obtained for the cultures corresponding to the growth curves shown in Figure 2.2. The red bar designates the growth rate obtained for glucose, the reference carbon source.

2.3.2.2. Screening for prebiotic candidates - remaining carbohydrates

The results of the growth experiments of the remaining carbohydrates (Table 2.2) demonstrate that only a limited number of carbohydrates (galacto-, isomalto-, malto- and soy-oligosaccharides, #23-27, 8-9, #18 and #33-34, respectively in Table 2.2) gave rise to higher cellular yield after 24 h than glucose, the reference carbon source. Other carbon sources that performed moderately well

consisted of (in decreasing order) are melibiose, raffinose, and XOS (# 29, 28 and 35-36 in Table 2.3, respectively). The fructans FOS and inulin, which are repeatedly regarded as bifidogenic carbohydrates, gave rise only to an OD value of ~2 after 24 h. The significantly higher cellular yield obtained on GOS compared to most of the other carbohydrates tested could be explained by its 17.5% w/v content of protein, thereby supplying the bacterium with additional source for peptides.

Relating the results of the growth experiments to the current knowledge of prebiotic-related proteins and enzymes in the genus *Bifidobacterium*, as well as to the genome sequence of BB-12 (Gariggue *et al*. 2010), can supply some insights into the carbohydrates that support the growth of BB-12. Relevant enzymes and proteins identified and characterised from other bifidobacterial strains are listed as well. A few of the substrates that seem promising as growth stimulators for BB-12 were be further investigated via proteome and/or transcriptome analyses of samples isolated from cultures cultivated on these carbon sources.

2.3.3. Fructans

2.3.3.1. Putative fructan-active proteins in BB-12

To predict which genes/proteins are expected to be up-regulated upon growth on FOS and inulin, the genome of sequence of BB-12 was searched for genes that code for proteins related to the utilisation of these fructans. The results of this search showed that the bacterium possesses two putative gene clusters that may play a role in transport and metabolism of oligofructans (Fig. 2.5). The first putative gene cluster consists of a regulatory protein (BIF_00490), a sucrose-6-phsophate hydrolases (formerly annotated as β-fructofuranosidase; BIF_01865) and a pair of transport proteins (sucrose permease [BIF_00489] and BIF_00743). An ORF of approxiametly 200 nucleotides in length that is located between the genes coding for BIF_00489 and BIF_00490 is coding for a hypothetical protein (BIF_01864). The second putative fructan operon includes a ribose repressor (BIF_01423), sucrose phosphorylase (BIF_02090) and a transport protein of the MFS superfamily (BIF_01422).

A sucrose utilisation gene cluster with an identical organization with respect to three of the four genes in the above-mentioned predicted fructans/raffinose operon was identified in *B. animalis* subsp. *lactis* (Trindade *et al*. 2003). This cluster encodes a sucrose phosphorylase (ScrP), a GalR-LacI-type transcriptional regulator (ScrR), and a sucrose transporter (ScrT). Recombinant ScrP was found to be induced by sucrose, raffinose, or oligofructose and repressed by glucose. The enzymatic activity of a β-fructofuranosidase detected in permeabilised cells of *B. animalis* MB 103 was more than 5-fold higher upon growth on FOS compared with glucose (Rossi *et al*.

Table 2.2. Chemical structure and performance in supporting the growth of BB-12 of the tested carbohydrates.

	Carbohydrate	Chemical structure	Analysis method & scale	OD_{600} after 24h
	glocuse (MRS, ready-made broth, Difco Laboratories, Detroit, MI.)	α-D-**glucose**	3×50 ml	5.0-5.7
	glocuse (Merck; dissolved in HM-MRS; reference carbon source)	α-D-**glucose**	3×50 ml	4.4
	Fructans			
1	Raftilsoe P95 (**FOS,** Orafti, Belgium)	a mixture of α-D-**glucose**-(1,2)-[β-fructose-(1,2-]$_n$ and [β-D-fructose-(1,2-]$_n$, n=2-8; **FOS** 93.2%; **glucose** + **fructose** + **sucrose** 6.8%	3×50 ml	1.8; 2.1[2]
2	Fibrulsoe 97 (**FOS**, Cosucra S.A, Belgium)	α-D-**glucose**-(1,2)-[β-fructose -(1,2-]$_n$ n=2-9	2×10 ml	1.9-2.3
3	Beneo Synergy1 (FOS-enriched Inulin, Orafti)	Inulin (α-D-glucose-(1,2)-[β-D-fructose-(1,2-]n n=2-60) 90-94%; glucose + fructose + sucrose 6-10%	3×50 ml	1.5-2.4
4	1:1 w/w mixture of **inulin** (#3) and **FOS** (#1)	See #1 & #3	3×50 ml	1.6
5	fibruline XL (99% **inulin,** Cosucra S.A.)	α-D-**glucose**-1,2[β-D-fructose-(1,2)]$_n$; n > 10	2×VC[7]	n.g.o.[3,4]
6	Fibruline Instant (90% **inulin,** Cosucra S.A.)	not disclosed by the manufacturer	contains microbes	
7	**FOS** (95%, Qingdao Reach International, China)	a mixture of different DP[6] **FOS**: 95%; **glucose** + **fructose** + **sucrose** 5%; Predominant DP: 3-5	2×10 ml	3.5
	Glucans			
8	IMO 900P (**isomalto**-OS[1], Hayashibara, Japan)	[α-D-**glucose**-(1,6)-]$_n$ n=2-5; **IMO** 85%; **isomaltose** 10-27%; **isomaltotoriose** (5-15%)	2×10 ml; 3×100 ml; 1×250 ml	5.2-6.6
9	(IMO) IsoMalto-OS 90% (Qingdao)	**isomalto**-oligosaccharides 90%	2×10 ml	6.5
10	Premidex (**dextrin,** AMD,USA)	randomized **glucose** linked starch	2×10 ml	1.9; 6.5[4]
11	**Cyclodextrin** (Sigma)	[α-D-**glucose**-(1,4)]$_n$, n=6-8	2*×10 ml	n.g.o.
12	Oat **β-glucan** (NSI, USA)	a mixed-linkage (1,3), (1,4)-β-D-**glucan** 10% β-**glucans**; **cellulose** [(1,4)-β-D-**glucose**]$_n$ and magnesium stearate (filling and lubricating agent)	VC	7.23[5]; 6.6[4]. 5.4[5]
13	Nutriose	**Dextrin**		0.85[2]
14	Glucagel Barley **β-glucan** (GraceLinc, NZ)	(1,3)/(1,4)-β-D-**glucan** 83.2%; **starch** 6%; protein 4.1%; fat 1.1%	2×10 ml	n.g.o.
15	Barley **β-glucan** (Birte Svensson, privat collection)	(1,3)/(1,4)-β-D-**glucan**	VC	5.1[4]
16	High **amylose starch** (Penford, USA)	starch enriched with the linear poly[α-(1,4)-D-**glucose**] backbone	VC	n.g.o.
17	mechanically modified potato **starch** (Penford, USA)	not disclosed by the manufacturer	VC	n.g.o.
18	**Malto**-OS (Qingdao)	**malto-OS** 60% [α-D-**glucose** (1,4)-α-D-**glucose**]$_n$;	2×10 ml	7.3
20	**Palatinose** (Senn chemiacal, Switzerland)	6-*O*-α-D-**gluco**pyranosyl-D-**fructo**furanose	2×10 ml	n.g.o.
21	**Sorbitol** (chew-tech)	Hexane-1,2,3,4,5,6-hexaol (reduced **glucose**)	2×10 ml	n.g.o.

22	**Cellobiose** (Senn chemiacal, Switzerland)	4-*O*-β-D-**gluco**pyranosyl-D-**glucose**	2×10 ml	n.g.o.
	Galactans			
23	Vivinal **GOS** (Frieslands Foods, the Neatherlands)	**TGOS** (trans-**galacto**oligosaccharides with β-(1,4) glycosidic bonds and β-(1,6) glycosidic bonds) 28%, mono and di-saccharides 46.5%; protein 17.5%; minerals 3.1%; fat 1% and moisture 4%.	3×50 ml	5.8
24	Vivinal **GOS** syrop (Frieslands Foods)	75% w/v of #22		4.3[2]
25	**GOS 70% (Qingdao)**	**GOS** 40%; **lactose** 35-40%; **glucose** 20-25%; **galactose** 1%	2×10 ml	4.6; 3.6[2]
25	**GOS 70% (Qingdao)**	**GOS** 40%; **lactose** 35-40%; **glucose** 20-25%; **galactose** 1%	2×10 ml	4.6; 3.6[2]
26	Herba Lycopi (Chinese herb) **GOS** (Qingdao)	**GOS** (Verbascose [**galactose**-(1,6)]3-(1,6)-*O*-α-D-**glu**-(1,2)-β-D-**fructose]** + **stachyose** [β-D-**fructose**-[*O*-α-D-**galactose**-(1,6)]$_2$-(1,6)-α-D-**glucose]** + **raffinose** 72 %;	2×10 ml	4.8
27	Bi²muno (trans-**GOS,** Calsado,UK)	**TGOS** (β-(1,4) and β-(1,6) linked galactans) 51.8%; **GOS**: β-(1,3) 26%; β (1,4) 23%; β (1,6) 51%; DP2: 52%: DP3: 26%; DP4: 14%; DP5: 8%; **lactose** 27.9%; **glucose** (including maltodextrin) 9.1%; **galactose** 5.6%; **Gum Arabic** 4.4%; Proteins 1.2%; Moistrure 3%	1×100 ml	6.2; 6.0[2]
28	**Raffinose** (sigma)	α-D-**galactose**-*O*-α-D-**glucose**(1,6)-β-D-**fructose**	3×50 ml	2.7
29	**Melibiose** (Sigma)	α-D-**galactose**-α(1,6)-D-**glucose**	22×10 ml	4.2-4.7
30	**Lactulose** (Sigma)	4-*O*-(β)-D-**galacto**pyranosyl-D-**fructo**furanose	2×10 ml	n.g.o.
31	**Pectin** (Sigma)	esterified D-**galacturonic acid**	2×10 ml; VC	n.g.o.
32	**Lactitol** (Senn chemiacal, Switzerland)	4-*O*-β-D-**galacto**pyranosyl-D-**glucitol**	2×10 ml	n.g.o.
33	**Stachyose** (Qingdao)	β-D-**fructose**-*O*-α-D-**galactose**-(1,6)-*O*-α-D-**galactose**-(1,6)-α-D-**glucose**; **stachyose** + **raffinose** 80%	2×10 ml	5.3
34	**Soy** OS (Qingdao)	**stachyose** + **raffinose** 80%	2×10 ml	5.5
	Xylans			
35	**XOS** 95P (Shandong Longlive Bio-Technology, China)	**XOS** 95%; D-**xylose** and D/L-**arabinose** 5%; [β-D-**xylose**-(1-,4)]$_n$; n=2: 30%; n=3: 28%: 4<n<=7	2×10 ml	3.7; 3.6[2]
36	**XOS** (Qingdao)	**XOS** 95%; [β-D-**xylose** -(1,4)]n, n=2-7	2×10 ml	4.3; 3.7[2]
37	**Xylitol** (uknown manufacturer)	1,2,3,4,5-**pentahydroxypentane**	2×10 ml	n.g.o.
38	Rye flour **Arabinoxylan** (Megazyme, Ireland)	**arabinose** 38%; **xylose** 59%; other sugars 3%.	VC	n.g.o.[5]
Mannans				
39	Partially hydrolised **Guar gum** (Novartis Nutrition, U.S.A.)	soluble, shorter chains then **Guar Gum**	2×10 ml; VC[4]	n.g.o.
40	CitriStim **Mannan**-OS (ADM, USA)	not disclosed by the manufacturer.	contains microbes[4].	n.g.o
41	**Manno**-OS, (Qingdao)	not disclosed by the manufacturer.	2×10 ml; VC[7]	n.g.o.
42	**Guar gum** (sigma)	(1,4)-linked β-D-**manno**pyranose backbone, branched by a (1,6) to α-D-**galacto**pyranose); mannose/galactose = 1.5-2.0; up to 10,000 residues.	VC	n.g.o.[5]

***Itlaicized* font: carbohydrates that gave rise to an OD ≥ 2 after 24 h; 1. OS: oligosaccharides; 2. a single 10 ml experiment; 3. n.g.o.: no growth observed; 4. tested by duplicated viable counts of cultures containing 0.5% w/v carbon source, which were plated on MRS-agar plates and recovered at t = 0 and t = 24 h; 5. The same experimental outline as in #4, with 2% carbon source. Values represent the ratio between the viable counts at t=0 and t = 24 h, respectively; 6. DP: degree of polymerization, i.e. number of monomers in the oligosaccharide structure; 7. VC, viable counts.**

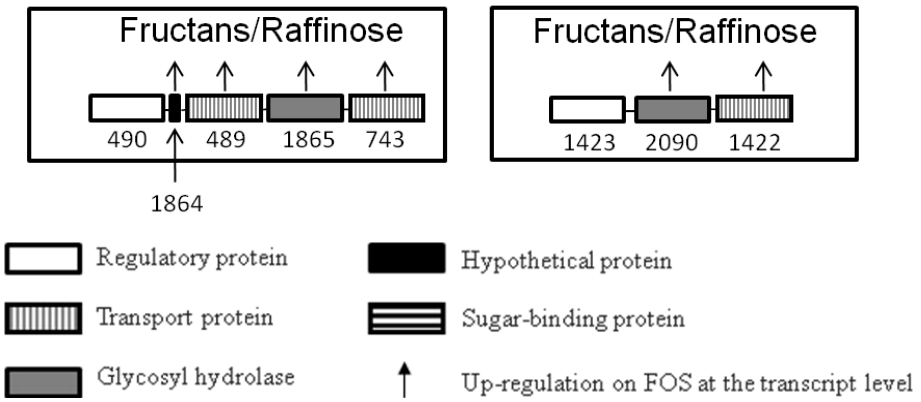


Figure 2.5. **Putative FOS/raffinose operons in the genome of BB-12. Numbers correspond to BIF locus tags.**

2005). An induction of β-fructofuranosidase during growth on FOS, sucrose and to a greater extent fructose, was observed in *B. infantis* ATCC 15697 (Perrin *et al*. 2001).

The genome sequence of BB-12 contains numerous genes coding for putative oligo- and polysaccharide-active proteins, yet the experimental data show that only a limited number of these proteins were up-regulated by FOS (compared with glucose; see section 3.2.2 below) at the transcriptional level, and none of them was induced at the translational levels (data not shown).

2.3.3.2. DNA microarrays analysis of BB-12 cultures grown on FOS

RNA samples derived from BB-12 cultures cultivated on FOS were subjected to comparative transcriptional analysis using DNA microarrays. 165 genes were up-regulated (fold change FOS/glucose > 2) and 140 genes were down-regulated (fold change glucose/FOS > 2; Table 2.3; Table A.1).

As expected, up-regulation on FOS was observed for most of the genes that constitute the two putative fructan operons (Table 2.3.A.) This trend is partially supported by the fact that in a similar analysis of BB-12 cultures grown on MRS medium supplemented with 1% w/v FOS (the same carbon source that was used in this study), the transcription of only two genes, BIF_01865 and BIF_00489, was induced. The fact that more genes were up-regulated on FOS in the present study may be explained by FOS being the primary carbon-source, in contrast to the earlier study where it was used as a supplement to a similar growth medium containing 2% w/v glucose. Parche and colleagues (2007) have also reported that homologous genes from B. longum NCC2705 were induced by FOS.

Three genes that code for proteins that may be associated with transport of oligosaccharides – MalG, MalC and a transporter, MFS superfamily (BIF_01618, BIF_01619 and

Table 2.3.A. Genes encoding putative fructan-acting proteins in BB-12.

Locus tag	Description	Fold-change	Organism where a homologous protein was described (reference) / additional information
BIF_00490	Transcriptional repressor	N.V.*	-*B. longum* NCC2705 (up-regulated on FOS; Parche *et al.* 2007)
BIF_01865	Sucrose-6-phsophate hydrolases (formerly annotated as β-fructofurano-sidase)	15	-*B. animalis* subsp. *lactis* BB-12 (Garrigues *et al*. 2005) -*B. animalis* subsp. *lactis* DSM10140 (Ehrmann *et al.* 2003) -*B. animalis* subsp. *lactis* (Trindade *et al.* 2003) -*B. adolescentis* G1 (Muramatsu *et al*. 1992) -*B. breve* UCC2003 (Ryan *et al*. 2005). -*B. longum* biotype *infantis* ATCC15697 (Warchol *et al.* 2002)
BIF_00489	Raffinose permease	18	-*B. animalis* subsp. *lactis* BB-12 (Garrigues *et al*. 2005)
BIF_00743	Transporter	2.1	-Conserved domain: periplasmic binding protein (PBP)b super family (cl11400), E value=3.1e-56
BIF_01423	Ribose repressor (formerly annotated as sucrose operon activator)	0.60	- *B. animalis* subsp. *lactis* (Trindade *et al.* 2003)
BIF_01473	Transporter, MFS superfamily	2.6	-homologous to sucrose transporter (ScrT) from *Bifidobacterium animalis* subsp. *lactis*, induced by sucrose and raffinose, repressed by glucose (Trindade *et al.* 2003)
BIF_01618	MalG	13	
BIF_01619	MalC	6.0	
BIF_02090	Sucrose phosphorylase	2.5	-*B. adolescentis* DSM20083 (Van den Broek *et al*. 2004) -*B. animalis* subsp. *lactis* (Trindade *et al.* 2003) -*B. longum* biotype *longum* SJ32 (Kim *et al*. 2003)
BIF_01422	Transporter, MFS superfamily (formerly annotated as sucrose-6-phsophate transporter ScrT)	6.0	-*B. animalis* subsp. *lactis* (Trindade *et al.*2003)

* No values obtained in the analysis

Table 2.3.B. Genes with putative role in complex carbohydrate catabolism that were up-regulated on FOS according to DNA microarrays analysis.

Locus tag	Description	Fold-change	Organism where a homologous protein was described (reference) / additional information
BIF_00132	1,4-α-glucan branching enzyme	4.6	EC 2.4.1.18
BIF_01252	β-Gal*f*-β-1,5-Gal*f* transferase/β-Gal*f* β- 1,6-Gal*f* transferase	2.3	EC 2.4.1.-/2.4.1.-
BIF_01579	β-D-Glc*p*-β-1,4-glucuronosyl-transferase	2.5	homologous to β-D-Glc*p* β-1,4-galactosyltransferase (EC 2.4.1.-; E value=1.70e^{-30}) from *Streptococcus thermophilus* (Stingele *et al.*1999)
BIF_01607	β-glucosidase (EC 3.2.1.21)	3.4	
BIF_01617	Pullulanase	7.5	
BIF_01864	Hypothetical protein	25	located in the middle of a putative FOS operon

BIF_01473, respectively; Table 2.3.A) were also up-regulated on FOS. The two former proteins, along with a gene coding for the starch-degrading enzyme pullulanase (BIF_01617) are located in tandem on the bacterial chromosome and may form another oligosaccharide-related operon (Fig. 2.7). MalG and MalC are homologous to the sugar permease of ABC transporter system from *B. longum* NCC2705 (BL00190 and BL0424, respectively). The former belongs to a FOS operon, the latter to an unknown operon (Parche *et al.* 2007), whereas BIF_01473 shows sequence similarity to the aforementioned ScrT. Since it is postulated that ABC-type transport systems for oligosaccharides may have broad substrate specificity (Parche *et al.* 2007), it is not unlikely that these proteins may also be involved in FOS uptake. The extensive up-regulation (fold-change=25) of the gene coding for hypothetical protein BIF_01864, for which no homologous proteins were found, may be a result of co-transcription, as this gene is located within the putative FOS operon (Fig. 2.5), whose constituents (except for BIF_00490) were up-regulated also during growth on XOS.

Six additional genes are predicted to be involved in complex carbohydrate metabolism (Table 2.3.B), yet they do not seem directly related to fructan utilisation. The remaining up-regulated genes (Table A.1, Appendix) consist of genes with a broad variety of putative functions, with high representation of ribosomal proteins.

With regard to the predicted mechanism for fructan catabolism in BB-12, the results of this study suggest that fructans are translocated across the cell membrane via oligosaccharide transporters of either an ABC or MFS type, and are degraded intracellularly by a sucrose-6-phsophate hydrolases (BIF_01865). The latter, which is also known as a β-fructofuranosidase (EC 3.2.1.26), hydrolyses the terminal non-reducing β-D-fructofuranoside residues in β-D-fructofuranosides as FOS and inulin. The products of this reaction, glucose and/or fructose (depending on whether the fructans, which are composed of backbone of fructose monomers linked through β-(2,1)-glycosidic bonds [the F_n type], which can alternativly contain a glucose moiety at the non-reducing end [GF_n type]), are then incorporated into the bifid shunt. Comparative sequence analyses of different bifidobacterial β-fructofuranosidases showed no secretion or membrane-anchoring sequences, suggesting that the enzyme may be acting intracellularly (Ryan *et al.* 2005) and that the bottleneck in inulin and medium-length sized FOS utilisation could be their import into the cell. This hypothesis is in line with the fact that BIF_01865 was not identified in 2D-DIGE analysis of the extracellular protein fraction of BB-12 (O. Gilad, unpublished data). In addition, the gene coding for this protein was highly up-regulated (15 fold) on FOS, as seen by DNA microarrays (Table 2.3.A).

2.3.3.3. Discussion

FOS and Inulin are widely used prebiotics, especially in Europe. Their performance in this study, however, was relatively poor. Cultures of BB-12 were propagated on both FOS, inulin or a mixture of the two attained more than 50% higher growth rates compared to glucose, yet the growth yields after 24 h were markedly lower (Fig. 2.2-2.4; Table 2.1). Similar growth patterns on FOS and inulin were observed for *B. animalis* DN-173 010 (Van der Meulen *et al*. 2004). The cultures grown on glucose, on the other hand, attained a lower growth rate, but grew continuously until the experiment was terminated after 24 h. This could be related to limited activity of the β-fructofuranosidase towards longer oligofructan chains. Another possible explanation is related to the structure of the oligofructans, some of which containing a glucose-$[Fructose]_n$ oligosaccharidic chains. If the glycosidic bond between the first and the second fructose monomers in the GF_n is cleaved first (it is however, unclear whether this cleavage is catalysed by the β-fructofuranosidase, which is not predicted to have an endo-mechanism, or by another glycoside hydrolases), the resulting sucrose, which is a preferred carbon source to glucose (data not shown), may be utilised first and when the cells run out of sucrose they will continue to degrade the FOS chain yielding free fructose monomers, a process that is evidently less energetically favorable then either sucrose or glucose utilization. The higher growth on sucrose and fructans (probably of the GF_2 and GF_3 type, which are composed of terminal glucose linked to two or three fructose monomers, respectively), can be explained by more efficient transport systems for di- and oligosaccharides compared with monosaccharides. Similar degradation patterns of FOS were reported for *B. infantis* ATCC 15697 (Perrin *et al*. 2001). The slightly higher growth rate obtained in the inulin-cultivated cultures compared with FOS may be related to the composition of the substrates, where the inulin mixture may contain higher fraction of GF_2 and GF_3 than FOS.

Comparable growth features were also observed by Rossi *et al.* (2005), who tested the growth of 55 bifidobacterial strains on FOS and inulin. Their study demonstrated that when *B. adolescentis* MB 239 was cultivated in a semi-synthetic medium supplemented with either FOS, glucose or fructose, the growth rates obtained on FOS were 3 or 4 fold higher compared with glucose or fructose (0.60, 0.19 and 0.15 h^{-1}, respectively). Similar specific growth rates to these values were obtained in the current study with BB-12 cultures grown on 2% w/v of either FOS, inulin or a 1:1 w/w mixture of the two (Fig. 2.2-2.4; Table 2.1).

The relatively poor growth of BB-12 on the long chain fructo-oligosaccharide inulin is also in agreement with the results obtained by Rossi *et al*. (2005). Among the 13 bifidobacterial strains of animal origin tested, only *B. thermophilum* ATCC 25866 grew on inulin, and HPLC-

based carbohydrate profile of the cultures throughout the course of the growth experiments showed that this strain is capable of degrading FOS up to 10-15 fructan monomers, while the longer chain fructans present in inulin remained intact (Fig. 2.6). It can therefore not be ruled out that the same utilisation pattern for fructans also applies for BB-12, yet this has to be verified by investigating the sugar profiles, as performed in the above-mentioned study (Rossi *et al*. 2005).

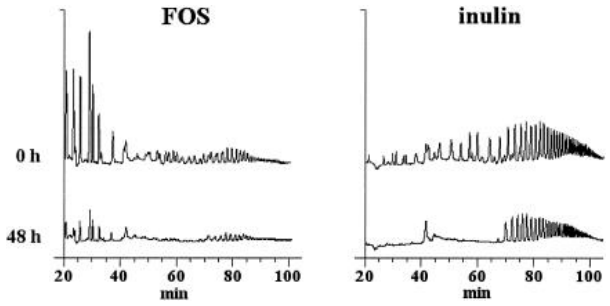


Figure 2.6 Comparison of HPAEC-PAD patterns of culture medium containing 10 g/l FOS or inulin at the inoculation and after 48 h of incubation with *B. thermophilum* ATCC 25866. The peaks correspond to the selectively eluted fructans with increasing DP. Adapted from Rossi *et al*. (2005).

A hypothetical explanation for the moderate stimulation of growth of BB-12 on oligofructans could be associated with the fact that the bacterium is only capable of utilising relatively short chain FOS, such that after these are hydrolysed extracellularlyor imported into the cell, the bacterium is incapable of utilising oligofructans of higher DP. It should be noted that BB-12 cannot ferment fructose, suggesting the lack of efficient transport system for this hexose. As a result, extracellular degradation of this substrate may result in accumulation of monomeric fructose at the exterior of the cell, yet the limited knowledge regarding the enzymes involved in FOS degradation makes these predictions very hypothetical.

Based on the up-regulation of several genes upon growth on FOS observed in DNA microarrays, some of the proteins predicted to play a role in fructan catabolism were identified. These predictions could be corroborated by characterisation of the aforementioned proteins and by knock-out studies. The results of this study indicate that despite of the well established documentation concerning the bifidogenic effect of FOS and inulin, these substrates do not seem to be very efficient growth stimulators for BB-12. One could, however, test the growth of the strain on FOS of lower DP (GF_2-GF_4), as it is possible that BB-12 can preferentially metabolise these oligosaccharides.

2.3.4. Galactans

2.3.4.1. Putative galactan-active proteins in BB-12

Since BB-12 was isolated from yogurt, where lactose is the primary carbon source, it is not surprising that within the putative operons associated with complex carbohydrate catabolism, GOS are the carbon sources for which the largest numbers of operons was predicted. Four putative galactan/raffinose operons consisting of 12, 10, 7 and 4 ORFs, respectively, are found in the genome of BB-12 (Fig. 2.7). With the exception of the latter, these gene clusters also contain genes with putative role in the utilisation of other sugars like *N*-acetylglucoseamine and gluco-oligosaccharides, as well as genes coding for predicted ABC- and MFS- type transporters, sugar binding proteins and both α- and β-galactosidases. The relatively high cellular yield obtained upon growth of BB-12 on GOS may be associated with the large number of β-galactosidases predicted to be involved in their degradation, as these enzymes are represented by as many as 8 ORFs in the genome of the bacterium (Fig. 2.7; Table 2.4). β-Galactosidases catalyse the degradation of the disaccharide lactose and galacto- and transgalacto-oligosaccarides, while α-galactosidases degrade melibiose, the trisaccharide raffinose and the soybean-derived oligosaccharide stachyose. The relatively large number of β-galactosidases may be related to complementary substrate specificity of the different enzymes, as shown for *B. bifidum* NCIMB41171 (Goulas *et al*. 2008).

2.3.4.2. Results and Discussion

Galactose-containing sugars were found to be relatively good substrates for the growth of BB-12 (Table 2.2). This applies especially for TGOS, GOS, soy-oligosaccarides and to a lesser extent also melibiose and raffinose. In addition, the specific growth rate attained for the GOS cultures was markedly the highest within the sugars for which the growth rate was calculated (Fig. 2.2–

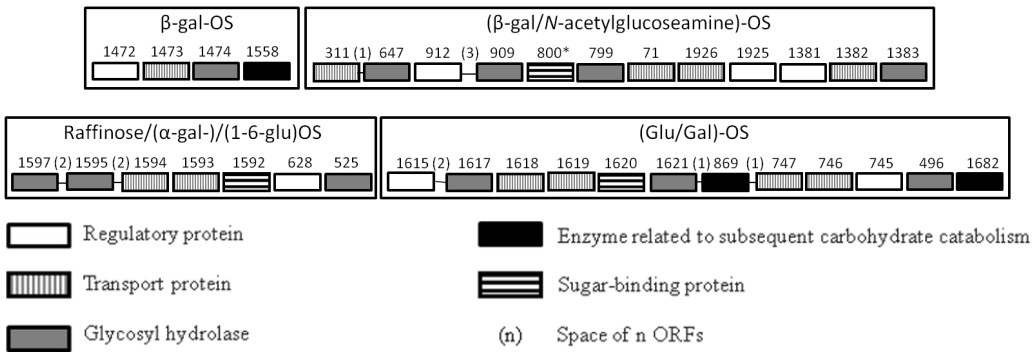


Figure 2.7. Putative gluco- and galacto-oligosacharides operons in the genome sequence of BB-12.

2.4), ans is yet supported by studies undertaken by Gopal *et al*. (2001) who reported higher growth of *Bifidobacterium lactis* DR10 on GOS compared to glucose, yet may be partly related to the protein content in the GOS powder.

Both α- and β-galactosidases were identified and characterised from a handful of bifdobacterial species (Table 2.4). Parche and colleagues (2007) predicted three potential lactose permeases in *B. longum* NCC2705, implying that this species confers a capacity to both degrade galactans and to transport them into the cell. It is still unknown, however, whether the degradation of the galacto-oligosaccharide chain takes place intra- or extracellularly.

Another interesting finding in this regard is related to the up-regulation of a putative ABC uptake system for raffinose when grown on solid agar-containing MRS medium in comparison with liquid MRS medium (Garrigues 2006). The expression of three genes of this uptake system was increased 51, 42 and 66 fold, for the two raffinose transport system permease proteins BIF_1592 and BIF_1593, as well as for the raffinose-binding protein (BIF_1594), respectively (Garrigues 2006). Agar is a seaweed-derived substance, consisting of a mixture of the galacto-polysaccharides, agaropectin and agarose. Noticeably, the genes coding for the *B. longum* NCC2705 proteins homologous to BIF_01592–94, which are predicted to belong to a raffinose operon with identical genetic organization, were over-expressed during growth on raffinose (Parche *et al*. 2007), suggesting that the BB-12 proteins encoded by the raffinose/α-GOS operon may be active against both agar and raffinose. The up-regulation of these raffinose-related genes, as well as the neighboring gene encoding an α-galactosidase (BIF_00525), which was induced 7-fold under growth on agar, is in agreement with the bifidogenic properties of GOS seen in the present study. In addition to that, some of the genes that embody the putative glu/gla-oligosaccharides operon – BIF_1617-19, were up-regulated on FOS according to DNA microarrays, implicating that this putative operon may have broad substrate specificity.

In comparison to the GOS, melibiose and raffinose gave rise to slower growth of BB-12. This could be explained by a possible switch in substrate utilisation; the galactose moieties are consumed first and the glucose monomers are utilised only after the depletion of galactose, as reported for *Bifidobacterium longum* NCC2705 (Parche *et al*. 2006). GOS, on the other hand, are in most cases exclusively composed of galactose moieties that may be preferably utilised by BB-12 compared to the glucose and GF (sucrose) moieties present in melibiose and raffinose, respectively. The higher specific growth rates observed in the GOS and raffinose cultures may be related to some lactose content in the GOS preparation, as it was shown that BB-12 grows faster on lactose than on FOS (Van der Meulen *et al*. 2006), yet is more likely to be associated with more efficient transport systems for oligo- and dissacharides compared with those utilized for

glucose uptake, as observed for both FOS, inulin and XOS (section 3.4).

With respect to glucose uptake, the identity of the proteins facilitating glocuse tranport is unclear. Possible candidates can be the MFS superfamily transporter, BIF_01334, which is slightly homologous to the D-glucose-proton symporter from *B. longum* NCC2705 that is repressed by lactose (Parche *et al*. 2006). No data was obtained for the expression of the gene encoding this protein in DNA microarrays analysis. Another putative glucose transporter may be the glucose uptake protein homolog (BIF_00684). The sequence of the latter is identical to the corresponding protein from *B. animalis* subsp. *lactis* DSMZ 10140, whereas the sequence of *B. animalis* subsp. *lactis* Bl-04 protein has a single SNP that diminishes the ability of the strain to grow on glucose, which is transported via facilitated diffusion (Briczinski *et al*. 2008). It is possible that this mechanism, which depends on the intra- and extracellular glucose concentration, is slower compared with the sugar-permease ATP hydrolysis-driven system predicted to be utilised for oligosaccharides. However, the expression of the gene coding for BIF_00684 was increased during growth on FOS compared to glucose, a finding that contradicts this hypothesis.

Table 2.4. Genes encoding putative GOS-acting proteins in the genome of BB-12.

Locus tag	Description	Organism where a homologous protein was described (reference) / additional information
-BIF_00311	Lactose permease	
-BIF_00489	Raffinose permease	
-BIF_00525	α-galactosidase	-*B. adolescentis* DSM 20083 (Leder *et al.* 1999; Van den Broek *et al.* 1999)
-BIF_01595*		-*B. breve* 203 (Sakai *et al.*1986)
-BIF_01624		-*B. longum* biotype *infantis* ATCC15697 (Roy *et al.*1992)
		-*B. longum* biotype *longum* CRL 489 (Garro *et al*. 1994)
-BIF_00647	β-galactosidase	-BIF_00647 is homologous to *lacZ of B. longum* NCC2705 that is up-
-BIF_00799		regulated by lactose, maltose and FOS (Parche *et al*. 2007)
-BIF_00909		-*B. adolescentis* DSM 20083 (Van Laere *et al.* 2000)
-BIF_01474		-*B. adolescentis* Int-57 (Lee *et al.*1997)
-BIF_01526		-*B. bifidum* A3 (Iwasaki *et al.* 1971)
-BIF_00909		-*B. bifidum* DSM 20082 (Dumortier *et al.* 1990)
-BIF_01952		-*B. bifidum* DSM 20125 (Møller *et al.* 2001)
-BIF_01953		-*B. longum* biotype *infantis* ATCC15697 (Roy *et al.* 1992)
-BIF_00944	Undecaprenyl-phosphate galac-	
-BIF_00983	tosephosphotransferase	
-BIF_01252	β-Gal*f*-β-1,5-Gal*f* transferase / β-	-EC 2.4.1.-/2.4.1.-
	Gal*f*- β-1,6-Gal*f* transferase	-up-regulated on FOS according to DNA microarrays
-BIF_01592	Raffinose-binding protein	

* Protein annotated as glycosyl hydrolases, formerly annotated as α-galactosidase.

2.3.5. Glucans

2.3.5.1. Putative glucan-active proteins in BB-12

Mining the genome sequence of BB-12 for gluco-poly- and oligo-saccharides-related genes resulted in the identification of two putative operons that were already listed with respect to GOS (Fig. 2.7; section 2.4.2.1.). It is unclear whether the oligo-1,6-glocosidase (BIF_01597) is co-transcribed with the GOS-related genes. In addition to these operons, a few additional genes (e.g. the 1,4-α-glucan branching enzyme [BIF_00132; Table 2.5] are not located in the vicinity of sugar-related genes.

2.3.5.2. Results

2.3.5.2.1. α-Glucans

It is evident that malto- or isomalto-oligosaccharides performed relatively well with respect to supporting the growth of BB-12 *in vitro* (Table 2.2) The gene locus *malERFG* of *B. longum* NCC2705, encoding a predicted maltose transport system, was induced when the bacterium was grown on maltose (Parche *et al.* 2007). The genome of BB-12 contains a gene coding for a maltose/maltodextrin-binding protein (BIF_00469) that possesses high sequence similarity to *malE*. In addition, the BB-12 genes coding for a transcriptional regulator of the LacI family (BIF_00745) and two multiple sugar transport system permease proteins (BIF_00746 and BIF_00747), are homologous with *malR*, *malF* and *malG*, respectively. Another relevant finding in this respect is the cloning and characterisation of the α-glucosidases encoded by the *aglA* and *aglB* genes from *B. adolescentis* DSM 20083. While the enzyme encoded by the former demonstrated high hydrolytic activity towards isomaltotriose and, to a lesser extent, towards trehalose, the enzyme encoded by *aglB* showed higher activity against maltose than sucrose and low activity towards maltotriose (van den Broek *et al.* 2003). In contrast to the growth-stimulating gluco-oligosaccharides and despite of the presence of genes encoding a variety of enzymes with putative activity against these substrates (Table 2.5), BB-12 is incapable of utilising a battery of α-glucans like potato starch, dextrin, cyclodextrin and the disaccharides cellobiose, palatinose and trehalose.

2.3.5.2.2. β-Glucans

The current *in vitro* growth studies demonstrated no growth of the bacterium on either oat or barley β-glucans, which may be related to the markedly lower number of β-glucosidases in the genome of BB-12 conmpared to α-glucosidases. β-glucosidases were identified and characterised from *B. breve* 203 (Sakai *et al*. 1986) and *B. breve* clb (Nunoura *et al.* 1996). The fact that β-

glucan did not show any bifidogenic properties with respect to BB-12 was also observed by Crittenden *et al*. (2002).

2.3.6. Mannans

Despite of the presence of two genes encoding a mannan endo-1,4-β-mannosidase in the genome of the bacterium, BB-12 showed no growth on the presence of either guar gum, a partially hydrolysed derivative of guar gum (PHGG), or two different preparation of manno-oligosaccharides (MOS). This is in agreement with an earlier study that reported that dietary MOS did not affect the cecal populations of bifidobacteria in ileal and cecal samples taken from turkeys

Table 2.5. Genes encoding putative gluco-oligosacchridic-acting proteins in the genome of BB-12

Locus tag	Description	Organism where a homologous protein was described (reference) / additional information
- BIF_00132	1,4-α-Glucan branching enzyme	-EC 2.4.1.18 -up-regulated by FOS according to DNA microarrays
- BIF_00151 **- BIF_00181** **- BIF_01617** **- BIF_02253**	Pullulanase	-*B. breve* strains JCM 7019, CCUG 43878, CCUG 34405, UCC 2003, and NCFB 2258 (Ryan *et al.* 2006, O'Connell-Motherway *et al.* 2008). - *B. dentium* NCFB 2243 (Ryan *et al.* 2006). -BIF_01617: up-regulated by FOS according to DNA micro-arrays
- BIF_0469	Maltose/maltodextrin-binding protein	-homologous to *malE,* which belongs *to* the gene locus *malERFG* from *B. longum* NCC2705 that is up-regulated by maltose (Parche *et al*. 2007). -homologous to *malE* from *E. coli* K-12, which binds malto-oligosaccharides (Quiocho *et al.* . 1997)
- BIF_00496 **- BIF_00782** **- BIF_02268**	amyB α-amylase; Trehalose-6-phosphate hydrolase	-*B. adolescentis* Int-57 (Rhim *et al*. 2006). -*B. adolescentis* DSM20083 (van den Broek *et al*.2003).
- BIF_00611 **- BIF_01373**	Isoamylase	
- BIF_00745	Transcriptional regulator, LacI family	
- BIF_00746	Sugar transport system permease protein	
- BIF_00747	MsmG	
- BIF_00869 **- BIF_01314**	4-α-Glucanotransferase	
- BIF_01597	Oligo-1,6-glocosidase	
- BIF_01607	β-glucosidase (EC 3.2.1.21)	-up-regulated on FOS according to DNA microarrays
- BIF_01618	Maltodextrin transport system permease protein malC	-up-regulated on FOS according to DNA microarrays
- BIF_01620	Maltose/malto--dextrin-binding protein	-homologous to the genes coding for the sugar ABC transporter solute-binding proteins BL1164 and BL1165 from *B. longum* LMG 13197, which are up-regulated by human milk oligosaccahrides (González *et al. 2*008). -up-regulated by FOS according to DNA microarrays -homologous to *malE* from *Streptococcus mutans* UA159, which binds malto-oligosaccharides (Webb *et al. 2*008).
- BIF_01621	Maltose transport system permease protein malG	
- BIF_01664	Thermostable β-glucosidase B	
- BIF_02090	α-Glucosidase	*B. breve* UCC2003 (Pokusaeva *et al*. 2009).

(Zdunczyk *et al.* 2005). Other studies, like those undertaken by Baurhoo *et al.* (2007a; 2007b), showed that MOS-containing carbohydrates gave rise to a significant bifidogenic effect ($P < 0.05$) in the ceca of in broilers. Furthermore, human studies demonstrated a significant increase in both the absolute values of fecal counts of *Bifidobacterium* and their proportion of the total viable counts during PHGG- and glucomannan-containing diets (Okubo *et al.* 1994; Chen *et al.* 2006).

2.4. Conclusions

2.4.1. Correlation between CFU/ml and OD_{600}

According to the linear correlation obtained in the study, OD_{600} value of 1 stands for 9.7×10^8 CFU of BB-12 per ml culture, which is in good agreement with the values described by Pérez-Consa *et al.* (2005). The correlation, after removing the outliers, was accompanied by a relatively low magnitude of inaccuracy (R^2=0.9704), and was independent of both the carbon source and the morphology of the cells.

2.4.2. Growth experiments

BB-12 was grown in the presence of a broad battery of carbohydrates, including fructans, galactans, glucans, mannans and xylans. The results suggest that galacto-, soy-, malto- and isomalto-oligosaccharides significantly stimulate the growth of the bacterium (both the growth rates and the absolute cell densities were higher than those obtained in cultures grown in the presence of glucose). XOS gave rise to mediate growth of BB-12, while fructo-oligosaccharides, (where the shorter fructans were favoured over for longer chain inulin or the mixture of the two) yielded relatively poor growth. Mannan-based carbohydrates like manno-oligosaccharides and guar gum did not result in any growth of BB-12, and neither did oat and barley β-glucan.

The relatively higher specific growth rate of BB-12 obtained upon cultivation on GOS, FOS, inulin, raffinose and XOS compared to glucose is likely to be related to utilization of transport systems for oligosaccharides of superior efficacy compared to glucose. It cannot be ruled out that the model for oligosaccharides transport described for a putative ABC-type transport system for XOS (Section 3.5) may also apply for the catabolism of other oligosaccharidic substrates.

The publication of the genome sequences of a few bifidobacterial strains - *B. longum* NCC2705 (Schell *et al.* 2002) and DJO10A, *B. adolescentis* ATCC15703, and of the *Bifidobacterium animalis* subsp. *lactis* strains DSM 10140, AD011, Bl-04, HN019, V9 and BB-12, has dramatically increased the knowledge of the metabolic capabilities of these members of the genus. The available sequences and the rapid development of advanced bioinformatics tools have facilitated the unraveling of the versatile metabolic toolbox possessed by bifidobacteria,

which encode a variety of glycosyl hydrolyses and sugar transporters. In this study, the genome sequences of BB-12 and *B. longum* NCC2705, as well as a review article on glycoside hydrolases (van den Broek *et al.* 2008b) served as the pillars of a comprehensive listing of prebiotics-related proteins in bifidobacteria, as illustrated in Tables 2.3–2.5 and 3.1–3.2.

It is noteworthy to stress out, however, that the presence of a gene in a genome of a given bacterium does not necessarily mean that the corresponding protein is indeed expressed and active. The aforementioned species-specific variation in the metabolic features of the bacteria should also be taken into consideration while comparing genes and proteins of different species.

Along with the results obtained in the transcriptomic and proteomic analyses performed on BB-12 cultures grown in the presence of a few selected prebiotic candidates, the results of the screening for growth stimulators for BB-12 are expected to increase the knowledge regarding the actual prebiotics-related metabolic capabilities of the bacterium (a detailed study of genes coding for XOS-active proteins, as well as a model for their utilization are described in chapter 3).

2.5. Materials and Methods

2.5.1. Experimental layout

The various prebiotic candidates were tested for their ability to support the growth of BB-12 at a concentration of 2% w/v, which is the glucose concentration in the commercial MRS medium commonly used for cultivation of lactic acid bacteria. The carbohydrates were tested either by biological triplicates in 50 ml tubes, duplicates in 10 ml tubes or by viable counts (see Table 2.2).

2.5.2. Preparation of selective growth medium

"Home-made"-MRS (HM-MRS) is a reconsistuated glucose-free medium based on the commercial MRS-B medium (Difco), whose components are depicted in Table 2.6. below. The pH of the medium was adjusted to 6.5 before sterilisation (210 kPa, 121°C, 20 min). Carbohydrate solutions were filtered through a 0.22 μm pore size filter and added to the sterile broth to obtain a final carbohydrate concentration of 20 g/l.

2.5.3. Correlation between CFU/ml and OD_{600}

A dataset of 20 data points, collected from three independent BB-12 cultures, was obtained by making duplicated dilutions of designated cultures. The OD_{600} values of the cultures was measured, 80 μl were plated out on Petri dishes containing MRS-agar supplemented with 0.05% w/v cysteine-hydrochloride (Cys-HCl), and the plates were incubated for 36 h in an anaerobic chamber supplemented with an anaerobic socket.

Table 2.6. The constituents of the HM-MRS medium.

Component	Conc. g/l
Tryptone	10
Beef extract	10
Yeast extract	5
Tri-ammonium citrate	2
Sodium acetate	5
Magnesium sulfate·$7H_2O$	0.1
Manganase sulfate·H_2O	0.05
Di-potassium phosphate	2
Tween 80	1 ml
pH	6.5

2.5.4. Preparation of crbon source solutions

Readily soluble carbohydrate (# 1-9, 18-22, 25-30, 32-37 and 41 on the carbohydrate list; Table 2.2) solution containing 2% w/v carbon source were prepared by dissolving the carbohydrates in autoclaved HM-MRS medium. The carbohydrate-medium mixtures were then sterile-filtered and placed in 37˚C overnight (ON), so that possible contamination could be detected and in order to pre-warm the medium to match the temperature the growth experiments were carried out at. Hardly-soluble viscous carbohydrates (the rest of the carbohydrates on the list) were weighed and dissolved in the pre-heated medium, and then set in 80˚C for 5 min, prior to the addition of the overnight inoculum.

2.5.5. Growth experiments

An inoculum of BB-12 was added into a tube containing MRS medium, into which Cys-HCl was added beforehand to a final concentration of 0.05% w/v, and the culture was incubated overnight at 37˚C. In the following morning, Cys-HCl (final concentration 0.05% w/v) was added to the carbohydrate solutions. To account for the turbidity originating from the carbon-sources, the OD_{600} of the solutions was measured (background OD) and the overnight inoculum was added to a final concentration of 1% v/v, yielding initial OD values that varied between 0.03 and 0.1 (from which the background OD was subtracted). The cultures were placed at 37˚C. The growth of the bacterium was monitored by OD measurements after 2, 4, 6 , 8, 12, 16, 20 and 24 h (for carbohydrates #1, 4, 5, 23, and 28) and to a lesser extent (OD measured after t=24 h) for the rest of the materials, which were only tested on a pilot basis. All the growth experiments were supplemented with cultures containing 2% w/v glucose (reference carbon-source), cultures into which no carbon source was added (designated as “HM-MRS”; negative control) as well as with cultures grown on MRS medium (validating that the bacterial inoculum grows as expected by culturing it on the medium the bacterium is usually cultured in).

2.5.6. Growth Rate Calculations

The carbohydrates that gave rise to viscous opaque solutions, making OD measurements unfeasible, were tested by viable counts. These were conducted by two consecutive dilutions of 80 µl of the cultures at t=0 with 9 ml peptone water, mixing and plating out in Cys-HCl containing MRS-agar plates, which were thereafter incubated anaerobically for approx. 36 h at 37°C. Both the dilutions and the plating were performed in duplicates, and the samples were designated as "10^-4". The same procedure was repeated after the cultures were incubated for 24 h, supplemented by an additional dilution 5-fold dilution ("10^-5"). The obtained dilution ratios were thus 12,822 and 64,411 for the "10^-4" and "10^-5" samples, respectively. The growth of the bacterium was estimated according to the ratio between the viable counts at t=24 and t=0.

Triplicate growth experiments

The specific growth rate of BB-12 in the presence of different carbon sources was calculated based on the OD_{600} measurements of 1 h intervals, from 2 h after incubation until 7 h, (the time range that gave rise to the best linear correlations, as calculated by a trial and error fashion). The calculations are based on the Malthusian growth model, which is commonly applied for microbial exponential growth: $N = N_0 \times 2^{\mu t}$, where µ is the growth rate, N is the cell count (or OD value), N_0 is the initial cell count at t=0 and t is the time. From here, the growth rate and the doubling time could be derived by plotting the $\log_{10}$ of the OD values as a function of time, such that a linear correlation is obtained in the exponential growth phase. The efficacy of the tested carbohydrates in supporting growth of BB-12 was evaluated based on the turbidity after 24 h incubation.

2.5.7. DNA microarrays analysis

2.5.7.1 Isolation of total RNA

At an OD_{600} of 0.87 and 0.62 (late-exponential growth; 8 h of growth) for the glucose and FOS samples, respectively, cultures were collected in RNA protect (Qiagen, Valencia, CA). Total RNA was isolated by using an RNeasy minikit (Qiagen) except for the following: 200 µl of TE buffer contained 15 mg/ml of lysozyme (L6876, Sigma-Aldrich, Brøndby, Denmark), 15 µl of proteinase K (20 mg/ml, Qiagen), and 2 µl of mutanolysin (M9901; Sigma-Aldrich) at 25 U/µl. Vortexing was every 10 s at 37°C for 2 min. The quality and concentration of RNA was determined by using the RNA 6000 Nano Kit on a Bioanalyzer 2100 instrument (Agilent Technologies, Palo Alto, CA). RNA was eluted with 2 x 55 µl of nuclease-free water (NFW), and routinely 20 to 50 µg of total RNA was obtained. Only high-quality total RNA, as indicated by two sharp rRNA peaks, was used.

2.5.7.2. cDNA synthesis and labelling of total RNA

A total of 10 µg of total RNA was lyophilized in a Speed-Vac. The copying into cDNA and labelling with either Cy3 (reference) or Cy5 (test) was performed using the CyScribe post-labeling kit according to the manufacturer's protocol (Amersham Biosciences, Hillerød, Denmark). One microliter of random nonamer from the kit was used for priming.

2.5.7.3. Hybridization, washing, and scanning of arrays

According to the Amersham protocol, the Cy3- and Cy5-labeled cDNA were combined and lyophilized. Hereafter, they were resuspended in 3 µl of NFW, and 5 µl of hybridization buffer (4x) and 12 µl of formamide was added. This 20-µl solution was applied to the array under a 24-by-24-mm LifterSlip (Erie Scientific Company, Portsmouth, NH) and put in an airtight box containing paper towels soaked in NaCl-saturated water. Arrays were hybridized for 16–20 h at 42°C and then washed using the UltraGAPS protocol (Corning B.V.). Arrays were scanned by using a GenePix 4100A personal scanner with GenePix Pro 6.0 software (Axon Instruments, Inc., Union City, CA). The photo multiplier tube (PMT) sensitivity values of the scanner software were set to with “Auto-PMT” with a “saturation tolerance” of 0.1%.

2.5.7.4. Preanalysis of arrays

In GenePix Pro software, a grid with 110-µm spots was superimposed on the array image and manually adjusted to fit small irregularities in spotting. Spots clearly covered by, e.g., dust particles were flagged as "bad" (equal to a Flag value of –100). The intensities of all the spots were then calculated by using the "analyze" function. Spots with very weak signals in both channels were also flagged "bad." These were spots where the signal-to-noise ratio was < 1 in both channels or the percentage of feature pixels with intensities more than two standard deviations above the background pixel intensity was < 20, i.e., (% > B + 2SD) < 20, in both channels (see the GenePix Pro manual for details). All other spots were considered "found" and had a Flag value of ≥0. All data were exported from GenePix Pro in tab-delimited format. In further analyses, the variables of feature (spot) intensity minus the background intensity around the spot was used, i.e., (F635 median - B635) = Cy5 and (F532 median - B532) = Cy3. The$\log_2$(ratio) was calculated as $\log_2$(Cy5/Cy3). Genes were discarded if the standard deviation of the $\log_2$(ratio) of the replicate spots on each array was >0.5. Genes were considered to be regulated when a |fold change| > 2 was obtained.

Chapter 3. Combined transcriptome and proteome analysis of *Bifidobacterium animalis* subsp. *lactis* BB-12 grown on xylo-oligosaccharides and a model of their utilisation

3.1. Summary

Recent studies have demonstrated that xylo-oligosaccharides (XOS), which are classified as emerging prebiotics, selectively enhance the growth of bifidobacteria in general and of *B. animalis* subsp. *lactis* strains in particular. To elucidate the metabolism of XOS in the well-documented and widely-used probiotic strain *B. animalis* subsp. *lactis* BB-12, a combined proteomic and transcriptomic approach was applied, involving DNA microarrays, real-time quantitative PCR (qPCR) and two-dimensional difference gel electrophoresis (2D-DIGE) analyses of protein extracts obtained from cultures grown on either XOS or glucose. The analyses show that 9 of the 10 genes that encode proteins predicted to play a role in XOS catabolism (*i.e.* XOS-degrading and metabolising enzymes, transport proteins and a regulatory protein) were induced by XOS at the transcriptional level, and the proteins encoded by three of these (β-D-xylosidase, sugar-binding protein, and xylose isomerase) showed higher abundance on XOS. Based on the obtained results, a model for the catabolism of XOS in BB-12 is suggested, according to which the strain utilises an ABC transport system (probably for oligosaccharides) to bind XOS on the cell surface and transport them into the cell. XOS are then degraded intracellularly through the action of xylanases and xylosidases to D-xylose, which is subsequently metabolised by the D-fructose-6-P shunt. The findings obtained in this study may have implications for the design of a synbiotic application containing BB-12 and the XOS used in the present study.

3.2. Introduction

Prebiotics are defined as food components that confer a health benefit on the host through modulation of the microbiota (WHO/FAO 2007). Among different kinds of prebiotics, special focus has been given to non-digestible oligosaccharides (NDOs), which can be fermented by the majority of the members of the genus *Bifidobacterium*. Bifidobacteria degrade NDOs to monosaccharides, which in turn are converted into intermediates of the bifid shunt (Section 1.1.1).

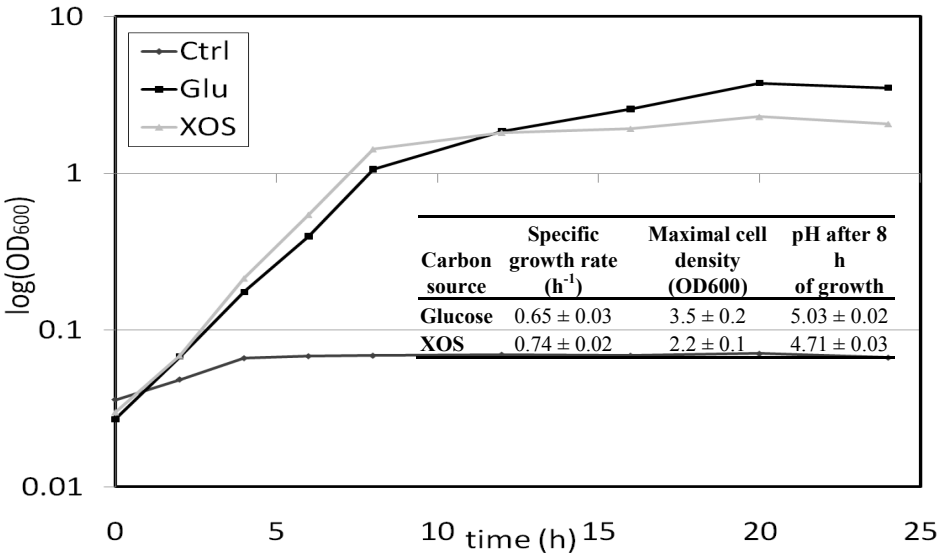
The most predominant sugar transport systems in bifidobacteria are ABC-type transport systems (Section 2.2). The capability of these transport systems to import di- and oligosaccharides is in line with the observation that some bifidobacteria obtain higher cell yields and higher specific growth rates when cultivated on oligosaccharides, compared to the corresponding monosaccharides

(Van der Meulen *et al*. 2004). These observations may be explained in part by efficient transport systems specific for di- and oligosaccharides.

Xylo-oligosaccharides are NDOs that have received increasing attention as potential prebiotic candidates (Roberfroid 2007). XOS are sugar oligomers composed of a β-1,4-linked xylopyranosyl backbone that are obtained by either chemical, or more commonly, enzymatic hydrolysis of xylan polysaccharides extracted from plant cell wall. The bifidogenic effect of XOS was demonstrated both by *in vitro* studies (Rycroft *et al.* 2001) and by small scale *in vivo* human studies (Chung *et al*. 2007), and a recent semi-continuous, anaerobic colon simulator study demonstrated that growth on XOS can also result in decreased levels of pathogenic strains (Mäkeläinen *et al*. 2009b).

A few XOS-degrading enzymes have been identified and characterised in bifidobacteria. A β-D-xylosidase characterised from *B. breve* K-110 (Shin *et al*. 2003) was shown to hydrolyse xylan to xylose. In a study that tested the activity of arabino-XOS degrading enzymes in *B. adolescentis, B. infantis* and *B. bifidum*, all species demonstrated intracellular xylosidase and arabinosidase activities, whereas no xylanase activity was detected (Zeng *et al*. 2007). An exo-oligoxylanase (RexA) was recombinantly expressed and characterised from *B. adolescentis* LMG10502. This enzyme was acts at the reducing-end and hydrolyses birchwood xylan and oat spelt xylan (Lagaert *et al*. 2007).

B. animalis subsp. *lactis* BB-12 is a widely-used commercial probiotic strain isolated in 1983 by Chr. Hansen A/S. The strain is included in a variety of food applications and dietary supplements, and is ascribed various probiotic effects (Ouwehand *et al*. 2004). In the present study, BB-12 was cultivated in a rich broth supplemented with either XOS (DP 2–6) or glucose and the obtained samples were compared at both the transcriptional and the translational levels. To our knowledge, this is the first study comprising a comprehensive and complementary proteomic/transcriptomic approach in bifidobacteria. Based on the obtained results, a model was established for the fermentative catabolism of XOS by BB-12. The objective is to broaden the insight into the metabolic basis of the use of XOS as a supplement to probiotic preparations containing BB-12.



Carbon source	Specific growth rate (h^{-1})	Maximal cell density (OD600)	pH after 8 h of growth
Glucose	0.65 ± 0.03	3.5 ± 0.2	5.03 ± 0.02
XOS	0.74 ± 0.02	2.2 ± 0.1	4.71 ± 0.03

Figure 3.1. Growth of BB-12 in a rich broth containing 2% (w/v) glucose or XOS, respectively, as well as a control culture where no carbon source was added. Growth studies were carried out in triplicate, and a representative data set is shown. Note that without carbon source added there is only limited growth, which is presumably due to low amounts of other carbohydrates in the complex components of the MRS. Insert: mean values of specific growth rate, maximal cell density, and pH of culture supernatants after 8 h of growth are given.

3.3. Results

3.3.1. Comparison of growth on glucose and XOS

To test whether XOS can support the growth of *B. animalis* subsp. *lactis* BB-12, the strain was propagated in an MRS-reconstituted broth containing 20 g/l of either glucose or XOS for 24 h (Fig. 3.1). The mean specific growth rates, as calculated from the exponential-phase, were slightly higher for the XOS-grown cultures, yet growth on XOS was markedly impaired after 6-7 h, but less so for the glucose cultures was lower. Accordingly, the mean turbidity values of the glucose cultures measured after 24 h in the glucose cultures (3.5 ± 0.2) were markedly higher compared to XOS (2.2 ± 0.1). Similar experiments conducted with arabinoxylan or xylose resulted in very poor growth (data not shown). pH measurements after 8 h of growth showed that the decrease in pH from the initial 6.5 in the XOS culture (4.71 ± 0.03) was significantly larger compared with the glucose-containing cultures (5.03 ± 0.02). This is consistent with the faster growth, and thereby faster acidification, seen on XOS. No significant differences were observed in the levels of the primary end products of the bifid shunt – acetate and lactate (data not shown).

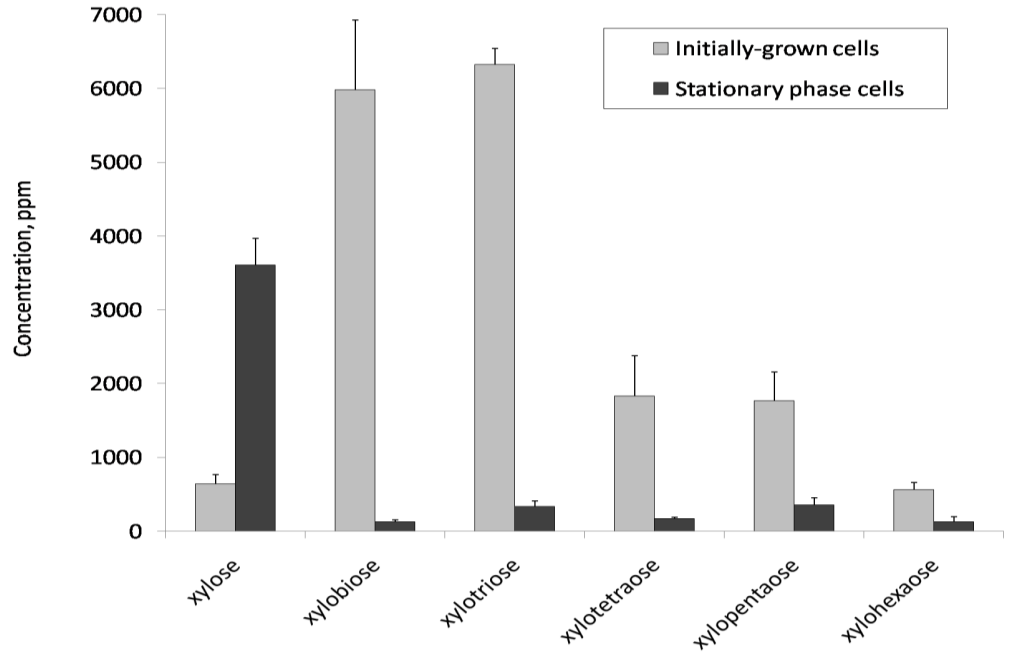


Figure 3.2. **High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis of supernatants of BB-12 cultures propagated on XOS. Concentration of different XOS species in the supernatants of BB-12 cultures grown on XOS collected from initially-grown cells and stationary cells, respectively, are designated. The results represent mean values and standard error from three biological replicates.**

3.3.2. Analysis of culture supernatants by liquid chromatography

Supernatants of BB-12 cultures grown in the presence of XOS were collected at the stationary phase (24 h) and subjected to High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis. The results (Fig. 3.2) showed that the concentration of all XOS (DP 2–6) was markedly lower at the stationary phase compared with initially-grown cells (93% decrease in total). The concentration of the monomeric D-xylose was nearly 6-fold higher, yet this accumulation accounted for less than 20% of the decrease in the total XOS conectration. The results also show that the relative decrease in the concentration of XOS of different DP decreased with increasing chain length (a decrease of 98, 95, 91, 80 and 77% for xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose, respectively). The level of arabinose in the above-mentioned culture supernatants was very low and the absence of glucose, galactose and lactose was by verified by gas chromatography analyses (data not shown).

3.3.3. DNA Microarrays analysis

The expression of genes from exponentially-growing BB-12 cultured in the presence of either glucose or XOS were compared using whole genome microarrays. Fourteen genes were found to be up-regulated and 10 to be down-regulated on XOS (Table 3.1). Of the genes induced upon growth on XOS, 11 are located successively in the genome of BB-12. Nine of these genes, along with another gene located elsewhere in the genome, encode proteins likely to be involved in XOS catabolism. These proteins consist of XOS-degrading enzymes, transport-related proteins, D-xylose-metabolising enzymes and a transcriptional regulator, which may play a role in the regulation of the genes (Table 3.2). Six of the 10 repressed genes (relative to growth on XOS) code for carbohydrate-metabolising or binding proteins, e.g. maltose/maltodextrin-binding protein (BIF_00469) and sucrose phosphorylase (BIF_02090).

3.3.4. Real-time quantitative PCR analysis

To verify the results obtained in the microarrays analysis, 11 putative XOS-related genes were subjected to qPCR analysis (Table 3.1). The induction of 9 of the 10 genes identified to be up-regulated in the microarrays analysis was confirmed by qPCR analysis. The exception was the transcriptional regulator BIF_00432. The correlation obtained between the up-regulation in the microarrays and qPCR analyses was good, yet the fold-change values were higher according to the microarrays analysis for 7 of the 9 genes found to be differentially-expressed in both analyses (Tables 3.1–3.2). No up-regulation at the transcript level was detected, however, for the gene coding for ATP-binding protein BIF_01681 that showed increased abundance at the protein level in cultures grown on XOS (Table 3.2).

3.3.5. Comparative proteome analysis using 2D-DIGE

Twenty-eight differentially abundant protein spots were identified in total (Fig. 3.4). The proteins in all of these spots were identified by MALDI-TOF MS/MS, amounting to 25 unique proteins (Table A.2, Appendix). Among these, 8 showed increased spot intensity and seven decreased abundance in the XOS cultures compared to the glucose-grown cultures. Three proteins were regulated more than five-fold – xylose isomerase (BIF_00501) and sugar-binding protein (BIF_00212) were up-regulated whereas enolase (BIF_01197) was down-regulated on XOS. Ten additional proteins were detected in 7 differentially abundant spots (6 of which were increased) whose analyses gave rise to more than a single protein identification, rendering the determination of the level of regulation impossible. Carbohydrate-metabolising proteins, including XOS- metabolising proteins and

Table 3.1. **Relative gene expression according to cDNA microarrays and qPCR analyses. Values of fold-change regulation (with glucose as the reference) for the microarrays analysis represent mean of two analyses carried out using a dye-swap, and values of the qPCR analysis are mean of 4 values from four biological replicates, each run in duplicates. Positive and negative values correspond to up- and down-regulation on XOS, respectively.**

ID	Description	Fold-change DNA micro-arrays	Fold-change qPCR
Up-regulated genes			
501	Xylose isomerase	33	19^{+++}
92	β-Xylosidase/α-L-arabinofuranosidase	31	10.3^{+++}
212	Sugar-binding protein	29	21^{+++}
257	Transporter	25	28^{+++}
258	Transporter	18	14^{+++}
405	β-Xylosidase/α-L-arabinofuranosidase	6.1	3.0^{++}
633	Endo-1,4-β-xylanase	6.0	2.1^{+}
1688	Hypothetical protein	5.9	n.a
829	Xylulose kinase	4.8	3.4^{+++}
1171	Sialic acid-specific 9-O-acetylesterase	3.7	n.a
1350	NrdI	2.2	n.a.
928	Endo-1,4-β-xylanase	2.2	2.5^{+}
1170	Para-nitrobenzyl esterase	2.1	n.a.
432	Transcriptional repressor	2.1	1.1
Unregulated genes			
1629	ABC transporter ATP-binding protein	1.2	0.82
1681	Sugar transport ATP-binding protein	1.1	1.1

ID	Description	Fold-change DNA micro-arrays	Fold-change qPCR
Down-regulated genes			
155	Formate acetyltransferase	-4.2	n.a.
1820	Hypothetical protein	-3.5	n.a.
2081	(S,S)-butane-2,3-diol dehydrogenase	-3.0	n.a.
800	Glycerol-3-phosphate-binding protein	-3.0	n.a.
1895	Inosine-uridine preferring nucleoside hydrolase	-2.8	n.a.
127	LSU ribosomal protein L31P	-2.6	n.a.
712	Pyruvate formate-lyase activating enzyme	-2.3	n.a.
2090	Sucrose phosphorylase	-2.2	n.a.
289	Multidrug resistance protein B	-2.2	n.a.
469	Maltose/maltodextrin-binding protein	-2.1	n.a.

$^{+}$ $P<0.05$; $^{++}$ $P<0.01$; $^{+++}$ $P<0.001$ (Anova one-way test); n.a. not analysed.

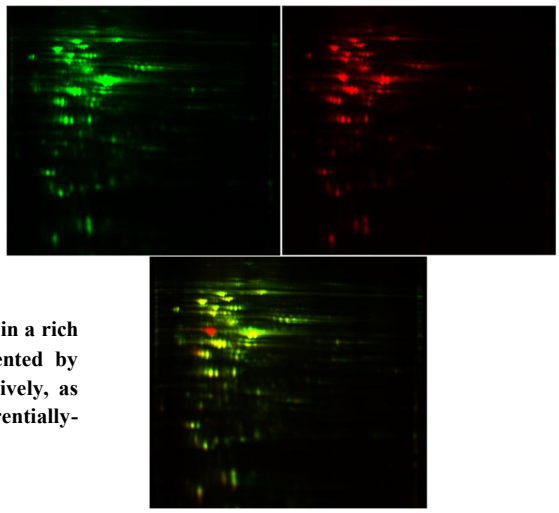


Figure 3.3. **Proteomic response of BB-12 to growth in a rich broth supplemented with glucose or XOS, represented by Cy3(green) and Cy5 (red) coloured images, respectively, as well as an overlay of the two images. Spots of differentially-abundant proteins are designated in Fig. 3.4 below.**

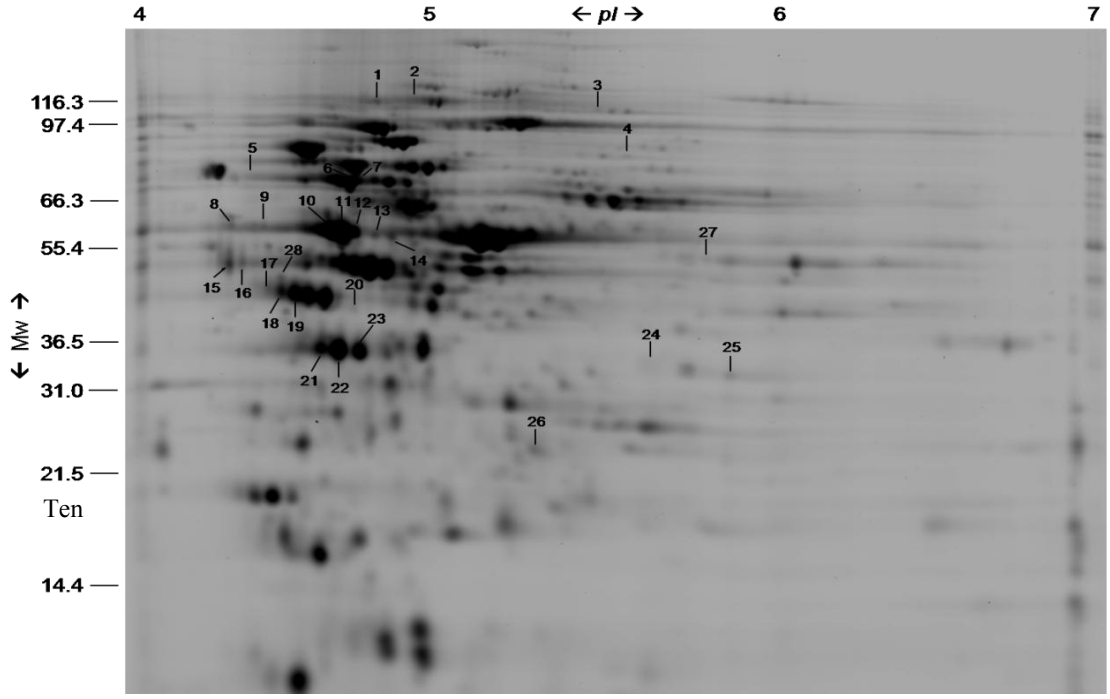


Figure 3.4. **Image of a representative 2D-DIGE gel. Differentially-expressed protein spots, molecular weight and isoelectric point markers are denoted. The proteins identified from the spots are listed in Table A.2 (Appendix).**

enzymes of the bifid shunt, accounted for the majority of the proteins identified from the 8 proteins whose abundance increased in the XOS samples. The results obtained from the proteomic and the transcriptomic analyses (Tables 3.1–3.2) demonstrate that proteins encoded by five of the 25 genes found to be regulated at the transcript level also had altered abundance at the protein level (yet only four of the 15 regulated proteins appeared to be of immediate interest with respect to XOS catabolism and thus tested by qPCR).

With respect to XOS metabolism, four of the 8 over-expressed proteins may be associated with the utilisation of XOS. The genes coding for three of these proteins – sugar-binding protein (BIF_00212; Fig. 3.5.A), xylose isomerase (BIF_00501; Fig. 3.5.B) and β-xylosidase (BIF_00405) were up-regulated also at the transcriptomic level during growth on XOS. In addition to that, the abundance of the ATP-binding protein BIF_01681 was increased when grown in the presence of XOS. Another ATP-binding protein, BIF_01629, was identified in a differentially-abundant spot also containing another protein. These two proteins may be involved in an ABC-type transport system that transports XOS across the bacterial cell membrane.

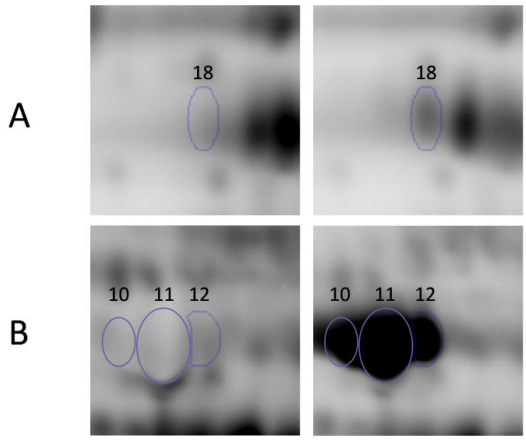


Figure 3.5. Comparison of the protein abundance in BB-12 grown on XOS or glucose (as observed by spot patterns on a gel image). Images (from a representative 2D-DIGE gel) were obtained by differential labelling of protein samples with fluorescent dyes. Spots A (spot #18) and B (spots # 10-12; see Fig. 3.4 and Table A.2, Appendix) correspond to sugar-binding protein (BIF_00212) and xylose isomerase (BIF_00501), respectively.

3.4. Discussion

In order to investigate the proteins and enzymes that play a role in the utilisation of XOS in the probiotic strain BB-12, comparative transcriptome and proteome analyses of samples derived from XOS-grown cultures were carried out. The results of the growth experiments showed that BB-12 attained slightly higher growth rate but lower growth yield on XOS compared to glucose. Earlier studies showed that BB-12 is capable of utilising XOS as a carbon source, but cannot ferment xylan, arabinoxylan (Crittenden *et al*. 2002) or xylose. Vernazza *et al.* (2006) reported markedly lower growth rate of BB-12 on XOS compared to glucose, yet comparison of growth rates and cell yields are potentially problematic due to differences in the growth medium and in the XOS used between the current study and the study carried out by Vernazza and colleagues. Cessation of growth was observed in the XOS-cultivated cultures after 6–7 h, which is probably related to the lower pH compared with glucose (4.71 and 5.03, respectively).

3.4.1. A model for XOS catabolism in BB-12

The induction of a few putative XOS-related proteins at the transcript and/or protein level supplied the basis for the establishment of a model for XOS catabolism in this strain. According to the suggested model (Fig. 3.6), the import of XOS and their further degradation take place in a multistep mechanism consisting of the following steps: (i) Binding of XOS at the cell surface; (ii) Transport across the membrane by ABC-type oligosaccharide transport system(s); (iii) Intracellular degradation of XOS to D-xylose by XOS-degrading enzymes; and (iv) Two-step conversion of D-xylose to D-xylulose 5-phosphate (X5P).

Table 3.2. Proteins/genes predicted to play a role in the catabolism of XOS in BB-12. Fold-change up-regulation values on XOS compared with glucose, as obtained using microarrays, qPCR and 2D-DIGE analyses are listed. Information regarding function predictions. For more detailed description of the protein hits see Table A.2 (Appendix).

Id[a]	Description	Fold-change up-egulation			Additional information[b]
		DNA micro-arrays	qPCR	2D-DIGE	
501	Xylose isomerase	32	19	13	- EC 5.3.1.5
405	β-Xylosidase	6.1	3.0	1.8	- EC 3.2.1.37/3.2.1.55 - GH 43 (3×10^{-36})
92	β-Xylosidase	31	10.3	n.d.[d]	- EC 3.2.1.37/3.2.1.55 - GH 43 (1×10^{-84})
432	Transcriptional repressor	2.1	1.1	n.d.	- cd01543, PBP1_XylR, ligand binding domain of DNA transcription repressor specific for xylose (1×10^{-15})
212	Sugar-binding protein	29	21	6.2	- COG1653, UgpB, ABC type sugar transport system periplasmic component (1×10^{-35}) - COG2182, MalE, Maltose binding periplasmic proteins/domains (2×10^{-17})
257	Transporter	25	28	n.d.	- PRK10999, malF, maltose transporter membrane protein (32%, 1×10^{-13}) COG1175, UgpA, ABC type sugar transport systems, permease components (7×10^{-58})
258	Transporter	18	14	n.d.	- COG3833, MalG, ABC type maltose transport systems, permease component (32%, 7×10^{-37}) - TC 3.A.1.1.21 xylobiose porter BxlEFG(K) (31%, 2×10^{-34})
633	Endo-1,4-β- xylanase	5.9	2.1	n.d.	- EC 3.2.1.8 - pfam04616, GH 43 (8×10^{-40})
928	Endo-1,4-β- xylanase	2.2	2.5	n.d.	- EC 3.2.1.8
829	Xylulose kinase	4.8	3.4	n.d.	- EC 2.7.1.17
1629	ABC transporter ATP-binding protein	1.2	0.82	1.8	- COG1129, MglA, ABC type sugar transport systems, ATPase component (7×10^{-6})
1681	Sugar transport ATP-binding protein	1.1	1.1	1.6	- COG3839, MalK, ABC type sugar transport systems, ATPase components (31%, 2×10^{-34}).

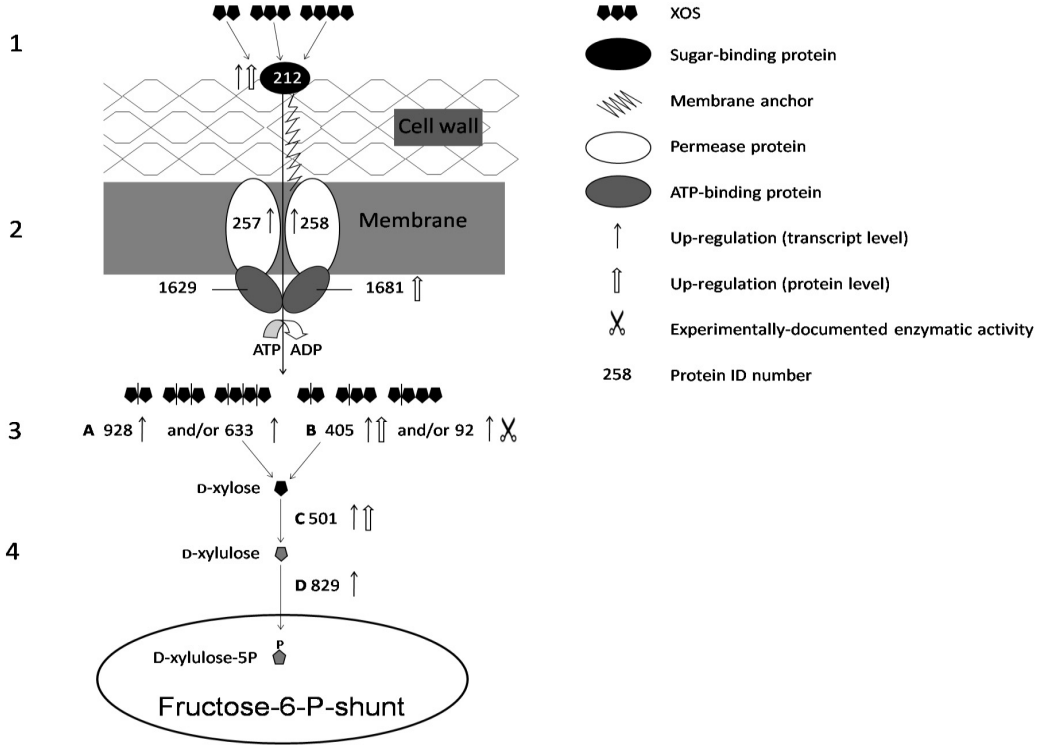
[a] Gene/protein number (the "BIF_" prefix was removed). [b] Sequence-based similarities to conserved domains, as predicted using BLASTP (Altschul *et al.* 1997) (expect values and amino acids percent identity are indicated in parentheses, respectively) and TCDB (TC numbers are indicated according to the TCDB classification system; http://www.tcdb.org/) (Saier *et al*. 2009). [c] Relevant organism(s) from which the proteins has been characterised and the corresponding reference. [d] n.d.: Proteins not detected to be up-regulated. For complete data of the analyses see Table A.2 (Appendix).

Binding of XOS at the cell-surface is carried out, according to the suggested model, by the sugar-binding protein BIF_00212, which is up-regulated on XOS at the transcriptional level. In the present study, the abundance of this protein was increased upon growth on XOS, and similar observations were recorded in extracellular proteome analysis (O. Gilad, S. Jacobsen, B. Stuer-Lauridsen and B. Svensson, manuscript in preparation).

Transport of XOS into the cells is predicted to be facilitated by an ABC-type sugar transport system(s) capable of importing a variety of oligosaccharides, as was postulated (at the genus level) by Palframan *et al*. (2003). The fact that nearly all of the XOS were consumed after 24 h or growth (Fig. 3.2) implies that the oligosaccharides were ta ken up by the cells.

The proteins predicted to take part in XOS transport are the transporter proteins BIF_00257 and BIF_00258. The transcription of the genes encoding these permeases was more than 20-fold up-regulated by XOS (Table 3.1). In addition to this, preliminary data show that both proteins were identified in the membrane fraction obtained from BB-12 cultures cultivated on XOS (Gilad *et al.* 2012). Sequence similarity searches of BIF_00257 and BIF_00258 (Table 3.2) demonstrate that these proteins resemble oligosaccharide transporters. The ATP-binding

Figure 3.6. A model for the catabolism of XOS in BB-12 comprising the following steps: (1) Binding of XOS at



the cell surface by a sugar-binding protein. (2) Transport of XOS by an ABC transport system. (3) Degradation of XOS to D-xylose (by a combined action of an endo-1,4-β-xylanase and a β-xylosidase). (4) Conversion of D-xylose to xylulose-5-P, a key metabolite of the fructose-6-P shunt. (A) Endo-1,4-β-xylanase. (B) β-Xylosidase. (C) Xylose isomerase. (D) Xylulose kinase. The numbers indicate the respective genes in the genome of BB-12, omitting the "BIF_0".

proteins that energise transport of XOS through the ABC transport system may be BIF_01629 and BIF_01681, the latter is differentially-expressed in cultures grown on XOS. According to the Conserved Domain Database searches (all non-redundant GenBank protein sequences at NCBI; http://www.ncbi. nlm.nih.gov/Structure/cdd/cdd.shtml) (Marchler-Bauer *et al*. 2009), these proteins

contain motifs characteristic of ATP binding sites.

The degradation of XOS transported across the membrane is suggested to be facilitated by the action of endo-1,4-β-xylanases (BIF_00633 and BIF_00928) and/or β-xylosidases (BIF_00092 and BIF_00405). Endo-1,4-β-xylanase cleaves XOS randomly, while β-xylosidase degrades the xylo-oligomeric chain at the non-reducing end, releasing D-xylose. The genes encoding these four enzymes were induced upon growth on XOS according to both DNA microarrays and qPCR analyses. According to the glycoside hydrolase classification system (http://www.cazy.org/Glycoside-Hydrolases.html) (Cantarel *et al*. 2009), BIF_00633, BIF_00092 and BIF_00405 are predicted to belong to glycoside hydrolase family 43 (GH43), which comprises both endo-1,4-β-xylanases and β-xylosidases. Preliminary data show that recombinant BIF_00092 degrades β-D-(1,4)-xylo-oligosaccharides of DP 2–5 (xylohexaose was not tested; A. H. Viborg, S. Jacobsen, K. I. Sørensen, O. Gilad and B. Svensson, manuscript in preperation). The up-regulation of BIF_00405 on XOS according to 2D-DIGE analysis provides additional experimental evidence supporting the suggested role of the two xylosidases in XOS degradation.

The final step in the model for XOS catabolism comprises conversion of D-xylose (formed by the action of the XOS-degrading enzymes) to X5P, as described for *Lactobacillus pentosus* MD353 (Lokman *et al*. 1997). D-Xylose is isomerised by xylose isomerase to D-xylulose, which is phosphorylated by xylulose kinase to X5P (Fig. 3.6). Xylose isomerase was highly up-regulated at both the protein and transcript levels (fold-change 32, 19 and 13 for the microarrays, qPCR and 2D-DIGE analyses, respectively) in the XOS cultures.

The increase in D-xylose concentrations observed in the present study may be associated with two alternative mechanisms. The first is related to extracellular degradation of XOS. This hypothesis does not seem very likely, however, as BB-12 grows very poorly on D-xylose, but demonstrates relatively high growth rate on XOS, suggesting the lack of efficient uptake system for the monosaccharide. As a result, the uptake rate of D-xylose monomers released from the action of extracellular XOS-degrading enzymes is expected to be low and hence the growth of the bacterium will be diminished. In addition, none of the four XOS-degrading enzymes are predicted to possess neither secretion-related sequence motifs nor a membrane/cell wall anchor, such that it is unlikely that any of these enzymes act extracellularly, yet it cannot be ruled out that another extracellular enzyme is involved in XOS degradation, or that one the above-mentioned enzymes is secreted. The second possible explanation to the accumulation of D-xylose could be that this monosaccharide is released to the exterior of the cell via diffusion or export mechanisms. Since BB-12 attains relatively high growth rate on XOS, it is possible that the incorporation of xylose resulting from

intracellular XOS degradation into the bifid shunt generates a bottleneck, which gives rise to redundancy in the intracellular xylose levels. It is unknown whether an inhibition mechanism is involved in this process, yet excess of D-xylose may in this case be exported or diffuse outside the cells where its concentration is lower. Growth experiments with radiolabeled XOS may be useful in corroborating these hypotheses.

The established model for XOS utilisation in BB-12 is currently being subjected to verification by comparative proteome analysis of the membrane proteins of the bacterium, as well as by characterisation of enzymes predicted to participate in this fermentative pathway. The activity and importance of the proteins predicted to play a role in growth on XOS can be verified by knock-out studies.

3.4.2. Previous models for XOS catabolism

The suggested model for XOS metabolism in BB-12 is in agreement with the model for *B. adolescentis* LMG10502 proposed by Lagaert *et al*. (2007), who postulated that XOS degradation by this strain is initiated by transport of XOS into the cell by a MalEFG-type oligosaccharide transporter followed by intracellular hydrolysis by a reducing-end exo-oligoxylanase to xylose and xylobiose. The latter can be further degraded by β-xylosidase, as previously reported for *B. breve* K-110 (Shin *et al*. 2003). It can be hypothesised that BB-12 utilises a similar transport system that is capable of transporting a variety of di- and oligosaccharides, including XOS and maltose.

A somewhat alternative model for XOS utilisation by *B. longum* biotype *longum* was introduced by van den Broek and co-workers (2008a). According to this model, XOS are degraded extracellularly and imported into the cell through a concerted binding-cleavage-transport mechanism that involves cleavage by extracellular membrane-anchored endo- and exo-xylanases to D-xylose and L-arabinose, which in turn will be transported into the cell by monosaccharide transporters. (van den Broek *et al*. 2008a) . This model differs from the model described in the present study, where XOS are taken up by the cells via ABC-type transport system(s) and degraded intracellularly to D-xylose.

3.4.3. XOS as a prebiotic candidate

XOS are expected to be resistant to degradation by the human upper GIT and to reach the colon, where they are preferentially fermented by bifidobacteria. As the nutrients levels in the colon are scarce, a daily dose of a few grams of XOS can boost the growth of bifidobacterial strains like BB-12. An experimental evidence supporting this notion can be found in a human intervention study (Chung *et al*. 2007), where administration of XOS resulted in significant increase in bifidobacterial

populations (as analysed from human feces), an increase that was diminished after the administration was halted.

With regard to the potential applications of XOS in a synbiotic preparation combined with BB-12, the present results show that the bacterium possesses the metabolic capacity to utilise XOS as primary carbon source. Mäkeläinen and colleagues (2009b) tested the effect of XOS of varying DP on the microflora in the lower GIT using a simulated colon model. The study showed that supplying the simulator with different types of XOS (including the XOS used in the present study) not only triggered an increase in the total counts of bifidobacteria and lactobacilli, but also caused a reduction in the total counts of clostridia (and, to a lesser extent, of bacteroides). The authors also observed that upon growth on the XOS tested, the levels of short-chain fatty acids increased and the concentrations of branched-chain amino acids decreased, data that indicate a balanced microflora. Taken together, these results exemplify the potency of XOS as prebiotics that selectively stimulate the growth of lactobacilli and particularly bifidobacteria. Since XOS was specifically efficient in promoting the *in vitro* growth of the *B. animalis* subsp. *lactis* taxon, e.g. BB-12, a synbiotic preparation containing a combination of the two may be taken into consideration.

3.5. Materials and methods

3.5.1 Strain and growth medium

Bifidobacterium animalis subsp. *lactis* strain BB-12 (BB-12 is a registered trademark of Chr. Hansen A/S) was obtained from Chr. Hansen A/S (Hørsholm, Denmark). Glucose was purchased from Merck Chemicals (Darmstadt, Germany). XOS powder (DP 2–6) was obtained from Shandong-longlive (Qindago, China), and contained (according to HPAEC-PAD) 90% XOS (of which 3.7% accounted for xylose, 1.0% arabinose, 34.6% xylobiose, 36.6% xylotriose, 10.6% xylotetraose, 10.2% xylopentaose and 3.3% xylohexaose) and 10% mono- and disaccharides. The fermentation medium was MRS (de Man 1960) made from its individual components, except from glucose that was omitted. The pH of the medium was adjusted to 6.5 before sterilisation (210 kPa, 121°C, 20 min). Carbohydrate solutions were filtered through a 0.22 µm pore size filter and added to the sterile broth to obtain a final carbohydrate concentration of 20 g/l.

3.5.2 Growth experiments

Stationary-phase precultures in MRS medium (Oxoid, Basingstoke, United Kingdom) supplemented with 0.05% (wt/vol) cysteine-chloride were harvested by centrifugation (3,200×*g* for 10 min at 4°C) and washed in 5 ml 0.9 g/l sodium chloride. The glucose- or XOS-containing broths and a control without added carbon source were inoculated with the washed pre-cultures to an OD_{600} of 0.05.

Hereafter the inoculated media were divided in two (for duplicate measurements). Growth was monitored for 24 h by manual OD_{600} measurements. Growth experiments were carried out in 50 ml centrifuge tubes in a water bath at 37°C. These cultures were used for microarrays and qPCR analyses. Identical setups of samples (in three biological replicates) were harvested for 2D-DIGE and HPAEC-PAD analyses.

3.5.3. Liquid chromatography

The analysis was undertaken on supernatants of BB-12 cultures grown on XOS collected after 0, 3 and 24 h of growth (samples for pH measurements were collected after 8 h). Due to technical problems the samples at t=0 h could not be analysed. Since there was limited growth and relatively low cell density at t=3 h, the samples at this time point were used as an approximation to the XOS baseline values. Chromatography was performed using a Dionex AS3500 instrument (Dionex, Sunnyvale, CA) for separation of carbohydrates on a CarboPac PA-100 column (4×250 mm, p/N 43055), in combination with a CarboPac PA-100 guard column (4×50 mm). Chromatography was performed as previously described (Gullón *et al*. 2008), with the following modifications: Samples were diluted 1:20 in Milli-Q water, the analyses were carried out by using a step-wise gradient at a flow rate of 0.8 ml/min and a total analysis time of 40 min. D-Xylose (Sigma), xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose (Megazyme, Bray, Ireland) were used as internal standards for quantification. All analyses were made in triplicate.

3.5.4. DNA microarrays

The design of the 65-75mer oligonucleotides for the BB-12 whole genome microarray platform was done as described previously (Pedersen *et al*. 2008) on a draft genome sequence of BB-12 (56 contigs). The platform specifications are available at the NCBI Gene Expression Omnibus (GEO) under platform accession no. GPL10040. The completed genome sequence of BB-12 can be found under GenBank accession no. CP001853. Isolation of total RNA, RNA quality control, microarray spotting, cDNA synthesis and labeling of total RNA, hybridisation, washing and scanning of arrays and pre-analysis of arrays was done as described previously (Pedersen *et al*. 2008) with the following modifications: hybridization was performed with 60% instead of 40% (vol/vol) formamide due to the relatively high GC-content of BB-12; microarray scanning was performed with "Auto-PMT" (Photo Multiplier Tube) set to a "saturation tolerance" of 0.1% instead of user-set PMT settings; genes were discarded if the standard deviation of the $\log_2$(ratio) of the replicate spots on each array was > 0.5 rather than > 0.8; only genes where data was obtained from both the standard array and dye-swap array were included. The details are described at the NCBI Gene

Expression Omnibus (GEO) under the series accession no. GSE20322.

3.5.5. Quantitative real-time PCR

Primer sequences for genes analysed by qPCR expression assays were designed using the Primer3 software (27). cDNA synthesis was carried out as described previously (Pedersen *et al*. 2008) with a total RNA input of 675 ng and done in duplicates for each of the four biological replicates. The obtained cDNA samples were diluted 1:72.5 in nuclease-free water. qPCR was carried out on an ABI 7500 qPCR machine (Applied Biosystems, Foster City, CA) using SYBR Green mastermix (Applied Biosystems) with 465.5 pmol/µl cDNA and 6 pmol/µl of each primer pair. PCR conditions used were as described previously (Pedersen *et al*. 2008). At the end of each reaction, cycle threshold (Ct) was manually set at the level that reflected the best kinetic PCR parameters and the $2^{-\Delta\Delta Ct}$ method of relative quantification (Livak 2001) was used to obtain expression values. The five genes (out of 8 genes tested) that gave rise to the most consistent expression, as analysed by the GeNorm software (Vandesompele *et al.* 2002), were used for normalisation of the results. The relative expression of each gene represented a mean of the values obtained for each of the four replicates, each being run in duplicates. A gene was considered to be regulated when |fold change| > 2, $P < 0.05$ in a one-way ANOVA test.

3.5.6. Extraction of intracellular proteins

Cells for proteome analyses were harvested after 8 h of growth by centrifugation (3,200×*g* for 10 min at 4°C) at OD_{600} of 1.0 and 1.4 for the glucose and XOS cultures, respectively. Cell pellets were washed twice with 50 mM potassium phosphate buffer pH 7.0 and kept frozen until disruption by passage through a French press (Duragauge P1603-136, Struers, Denmark) at 8,270 kPa. Cytosolic protein fractions were obtained as described (31), and the determination of protein concentration was performed using the Popov method (Popov *et al*. 1975).

3.5.7. Two-dimensional difference gel electrophoresis (2D-DIGE)

Volumes corresponding to 50 µg protein from each of the samples (three biological replicates of cells grown on glucose or XOS, respectively), as well as an internal standard obtained by pooling aliquots containing 25 µg protein from each sample, were precipitated with chloroform/methanol (Wessel and Flügge 1984). Precipitates were dissolved in a buffer containing 15 mM Tris, pH 8.5 and 8 M urea. Protein samples were labelled with fluorescent cyanine dyes as previously described (Ruiz *et al*. 2009), with the exception that 4 nmol dye (rather than 400 pmol) was used to label each of the samples. The labelled internal standard and the corresponding labelled samples for each gel

were pooled and the volume was made up to 350 µl by addition of rehydration buffer (0.2% [v/v] carrier ampholytes, 0.4% [w/v] 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate [CHAPS] and 50 mM DTT). Isoelectric focusing was run using immobilised pH gradient strips (linear pH grade 4–7, 18 cm; GE Healthcare, Piscataway, NJ). Polyacrylamide gel electrophoresis was performed using 10% (wt/vol) polyacrylamide gels on an EttanTM DALT-6 Electrophoresis Unit (GE Healthcare) overnight at 1 W/gel until the dye front reached the base of the gel. Fluorescence gel images were acquired by a Typhoon 9410 Variable Mode Imager (GE Healthcare) at excitation and emission wavelengths of 488/520 nm (Cy2), 532/580 nm (Cy3) and 633/670 nm (Cy5) and a 100 µm resolution. Subsequent to fluorescence scanning, gels were stained using colloidal Coomassie Brilliant Blue (Rabilloud and Charmont 2000).

3.5.8. Image analysis

Analysis of the gel images was undertaken using Progenesis SameSpots software version 3.3 (Nonlinear Dynamics, Newcastle upon Tyne, UK). The gel images were aligned by automated calculation of alignment vectors after assigning 20–30 landmark vectors. The scanned gels were analysed by intra-gel (methodological variance) and inter-gel (biological variance) analyses. A threshold of 1.5-fold for spot volume ratio change (in cases where proteins were identified from more than a single spot, the highest fold-change values were considered) and an ANOVA statistical significance test ($P < 0.05$) were chosen to identify differentially-expressed protein spots, excluding spots giving rise to more than a single protein hit, as determined by mass spectrometry.

3.5.9. In-gel digestion and protein identification by mass-spectrometry

Differentially-expressed protein spots from the 2D-DIGE analysis were manually excised and subjected to in-gel tryptic digestion as described (Rosenfeld *et al*. 1992) with minor modifications (Zhang *et al*. 2007), excluding the reduction and alkylation steps (already performed in the equilibration step of the 2D-DIGE). Tryptic peptides were analysed by an Ultraflex II MALDI-TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany). Spectra were obtained as a summation of 10 individual spectra in positive reflector mode and externally calibrated using a tryptic digest of β-lactoglobulin (5 pmol/µl). Internal calibration was performed by using trypsin autolysis products and keratin contaminants that were subsequently removed from the peak list. The FlexAnalysis 3.0.96 and Biotools 3.1 software (Bruker-Daltonics) were used to analyse the recorded spectra. Protein identification was performed by using the protein sequence database program MASCOT (http://www.matrixscience.com) with the genome sequence of BB-12 (represented by the locus tag identifiers BIF_0XXXX that correspond to the GenBank accession no. CP001853) (Garrigues *et al*.

2010) as taxonomy entry. The following search parameters were used in all Mascot searches: carbamidomethylation and methionine oxidation were taken into account as fixed and variable modifications, respectively, tolerance of one missed cleavage, and a maximum error tolerance of 80 ppm and 0.7 Da in the MS and tandem MS data, respectively. No restrictions with respect to protein mass and p*I* were made. Protein/ion scores with $P < 0.05$ were considered significant.

Chapter 4. The extracellular proteome of *Bifidobacterium animalis* subsp. *lactis* BB-12 reveals proteins with putative roles in probiotic effects

4.1. Summary

Probiotics are live microorganisms that exert health-promoting effects on the human host, as demonstrated for numerous strains of the genus Bifidobacterium. To unravel the proteins involved in the interactions between the host and the extensively used and well studied probiotic strain *Bifidobacterium animalis* subsp. *lactis* BB-12, proteins secreted by the bacterium, i.e. belonging to the extracellular proteome present in the culture medium, were identified by 2-DE coupled with MALDI-TOF MS. Among the 74 distinct proteins identified, 31 are predicted to carry out their physiological role either outside the cell or on its surface. These proteins include solute-binding proteins for oligosaccharides, amino acids and manganese, cell wall-metabolizing proteins, and 18 proteins that have been described to interact with human host epithelial cells or extracellular matrix proteins. The potential functions include binding of plasminogen, formation of fimbriae, adhesion to collagen, attachment to mucin and intestinal cells as well as induction of immunomodulative response. These findings suggest a role of the proteins in colonization of the gastrointestinal tract, adhesion to host tissues, or immunomodulation of the host immune system. The identification of proteins predicted to be involved in such interactions can pave the way towards well targeted studies of the protein-mediated contacts between bacteria and the host, with the goal to enhance the understanding of the mode of action of probiotic bacteria.

4.2. Introduction

Bifidobacteria are anaerobic, non-gas producing, non-motile, non-sporulating and saccharoclastic Gram-positive bacteria that are highly represented in the microbiota of the human gastrointestinal tract (GIT) (Ventura *et al*. 2007_b). The genus *Bifidobacterium* comprises of a multitude of strains that have been assigned a variety of health-promoting (probiotic) properties mediated through interactions between bacterial cells in the human GIT and the cells of the host. Due to these beneficial effects (*e.g.* modulation of host immune responses and enhancement of the competitive exclusion of pathogens [Marco *et al*. 2006]), probiotic *Bifidobacterium* strains are included in a variety of food applications and dietary supplements (Masco *et al*. 2005).

The interactions that mediate probiotic effects have in some cases been reported to be facilitated by the action of proteins or peptides exported by (among others) bifidobacteria (Sánchez *et al*. 2010_b). These proteins are either secreted to the exterior of the cell or remain attached to the bacterial cell wall or membrane, as conferred by export-associated sequence motifs and/or cell wall anchoring motifs (Sánchez *et al*. 2010_b). Other secreted proteins are classified as cytosolic, but are *via* a hitherto unknown mechanism, displayed at the cell surface or being exported outside the cell. These proteins are also known as "moonlighting proteins" (Jeffery 2003).

In a preliminary study of *Bifidobacterium longum* NCIMB8809, 14 unique secreted proteins were identified, including three hypothetical solute-binding proteins (SBPs) of ATP-binding cassette (ABC) transporters, which are homologous to an invasion associated protein, as well as putative enzymes catalyzing cell wall turnover (Sánchez *et al*. 2008_b). Analysis of the cell envelope proteome of the same strain resulted in identification of 128 distinct proteins, including characteristic cell wall proteins, ribosomal proteins and a few moonlighting proteins that may be important for interaction with the host (Ruiz *et al.* 2009).

In the present study, extracellular proteins from the extensively used commercial probiotic strain *Bifidobacterium animalis* subsp. *lactis* BB-12 were identified by 2-DE followed by MALDI-TOF MS. Despite the differences in the biological conditions which the bacterium is exposed to under monoculture growth in a rich broth compared with growth in the GIT, the knowledge regarding proteins present in the extracellular proteome of the bacterium identified in the present study will increase the understanding of the molecular mechanisms of the possible interactions between the bacterium and the human host.

4.3. Results and Discussion

4.3.1. Identification of extracellular proteins from *Bifidobacterium animalis* subsp. *lactis* BB-12 culture supernatants

To shed more light on the proteins secreted by BB-12, a proteomic analysis of the extracellular fraction from the bacterial cultures was performed. A total of 124 protein spots were detected by 2-DE (Fig. 4.1), which is several fold more than in a similar study of *B. longum* NCIMB 8809 (Sánchez *et al*. 2008_b). Using MS, proteins were identified in 96 spots (four of which gave rise to statistically significant protein hits with sequence coverage ≤ 15%) corresponding to 74 unique proteins (Table 4.1 & A.2). The identification of 23 proteins from several spots on the gel may result from post-translational modifications that alter the M_r and/or the p*I* of proteins and hence their migration through the gel, as was previously reported for *B. longum* NCC2705 (Yuan *et al*.

2006). For 25 of the identified proteins, homologous proteins (50–90% sequence identity) were found in either the extracellular or the cell envelope fractions of *B. longum* NCIMB 8809 [6–7]. This similarity implies that BB-12 and NCIMB 8809 may share some common features with respect to the proteins that are in contact with the environment.

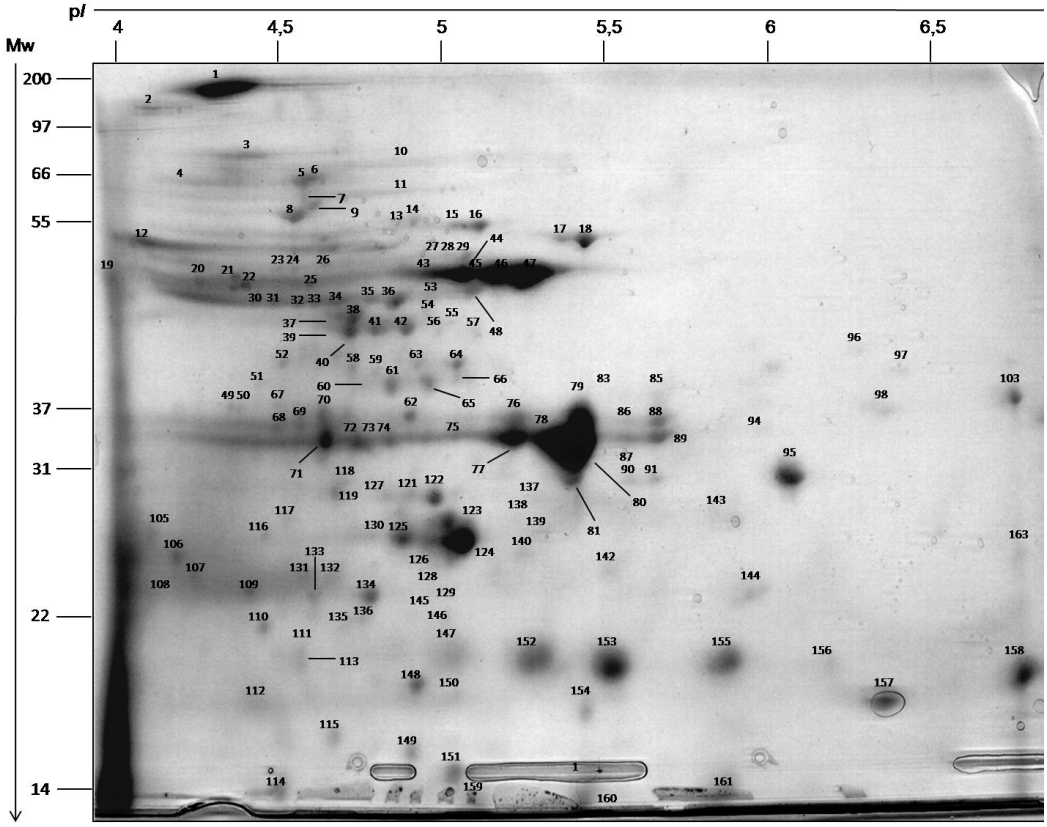


Figure 4.1. **A representative gel image of the extracellular protein fraction of *Bifidobacterium animalis* subsp. *lactis* BB-12. Molecular weight (Mw) and isoelectric point (p*I*) markers are denoted, as well as numbered protein spots corresponding to those listed in Table 7.3.**

4.3.2. Bioinformatic analysis of the identified proteins

Prediction of structural elements of the proteins identified in the present study, and of those of the predicted proteome of BB-12, showed that 10 and 14 proteins contained a signal peptide and TMSs, respectively. The secretion of these proteins is likely to take place by translocation across the cell membrane facilitated by the secA translocase (BIF_00806), which has been identified in the membrane fraction of BB-12 (Gilad *et al*. 2012). Eighteen of the 20 proteins predicted to be

subjected to non-classical (*i.e.* signal peptide-independent) secretion have been found to exhibit moonlighting functions in other bacteria, or may be involved in other non-cytoplasmic processes like cell wall metabolism and binding of sugars and amino acids, as indicated by sequence similarity searches.

Even though it cannot be ruled out that some of the proteins identified in the present study were detected extracellularly as a result of non-specific release of cytoplasmic components by cell autolysis, the 2-DE spots pattern of the secretome differs notably from the 2-DE pattern of the intracellular proteome analyzed under similar conditions (Fig. 4.2), as confirmed by software-based comparative image analysis (data not shown). This implies that the presence of cytoplasmic proteins in the culture supernatants was probably not primarily a result of autolysis and subsequent spill out of cytoplasmic proteins, but occurred to significant extent by selective secretion or passive diffusion of the proteins.

The COG functional classification of the identified proteins, and of the total proteome of the bacterium, respectively (Fig. 4.2), showed an overrepresentation of proteins classified within the categories "Post-translational modification, protein turnover, chaperones" (protein fate) and "Cell wall/membrane/envelope biogenesis" among the identified extracellular proteins. Whereas this is anticipated with regard to cell envelope-metabolizing proteins, the relatively large number of proteins involved in protein fate is surprising, but can partly be explained by the presence of chaperone proteins GROES, GROEL and DnaK (BIF_01614; the two latter are putative moonlighting proteins) predicted to have an extracellular role (Section 3.3.3.2), and two peptidyl-prolyl isomerases that may also act extracellularly. An expected underrepresentation of intracellular functions like transcription and replication was confirmed. The relatively high number of proteins that were not assigned to a category, or whose functional annotation was classified as general or hypothetical (18 proteins, nearly a fourth of the unique proteins identified) may be due to the limited knowledge of secreted and cell surface proteins in bifidobacteria.
assigned a cellular localisation). Another predicted cytoplasmic protein (hypothetical protein BIF_00480) includes a transmembrane domain.

Seven proteins were predicted to contain a signal peptide and their secretion is likely to take place via translocation across the cell membrane by the secA tranlocase (BIF_00806), which, along with other proteins associated with the tranlcation machinery, was also identified in the membrane fraction of BB-12 (Chapter 5). Twenty of the 27 proteins predicted to be subjected to non-classical *(i.e.* signal peptide independent) secretion were described to exhibit moonlighting

Table 4.1. **Predictions of subcellular localisation and structural features of proteins identified in the extracellular fraction of BB-12, compared with the total proteome**.

Subcellular component prediction*	Identified extracellular proteins (82)		Whole Proteome (1612)	
	number of proteins	% of total proteins	number of proteins	% of total proteins
Cytoplasmic	53	64.6	939	58.3
Membrane	11	13.4	422	26.2
Cell wall	2	2.4	8	0.5
Extracellular	2	2.4	9	0.6
Unknown	14	17.1	233	14.5
Structural features				
Proteins with TMD(s)†	14	17.1	400	24.8
Proteins with Signal pepetide§	7	8.5	69	4.3
Proteins secreted via non-classical secretion¶	27	32.9	465	28.8

* Predictions carried out using PSORTb 3.0.1; † The presence of transmembrane domain(s) (TMD(s)) was predicted with TMHMM; § Predictions obtained using SignalP 3.0; ¶ Predictions of performed with SecretomeP 2.0.

functions in other organisms or may have a non-cytoplasmic function, as indicated by sequence similarity searches.

The patterns of 2-DE spots obtained for the protein samples are accordingly notably different compared to the 2-DE patterns of intracellular proteins analysed under similar conditions (Fig. 4.2). The absence of DNA in the medium is supportive evidence for the notion that the presence of cytoplasmic proteins in the culture supernatant was due to a result of nonspecific release of cytoplasmic components by autolysis, but apparently occurred by selective secretion or passive diffusion of proteins. The COGs functional classification of the proteins identified and of the total proteome of the bacterium, respectively (Fig. 4.3), showed an overrepresentation of the identified extracellular proteins for proteins associated with posttranslational modifications, protein turnover and chaperones (protein fate) and proteins involved in cell wall/membrane/envelope biogenesis While this is not unanticipated with regard to cell envelope-metabolising proteins, the relatively large number of proteins involved in protein fate can be explained by the presence of two putative moonlighting chaperone proteins (GROEL and DnaK) that are predicted to possess an extracellular role, and three peptidyl-prolyl isomerases, which may act extracellularly. An expected underrepresentation of the intracellular cellular functions like transcription and replication was observed. The overrepresentation of proteins associated with nucleotide transport and metabolism

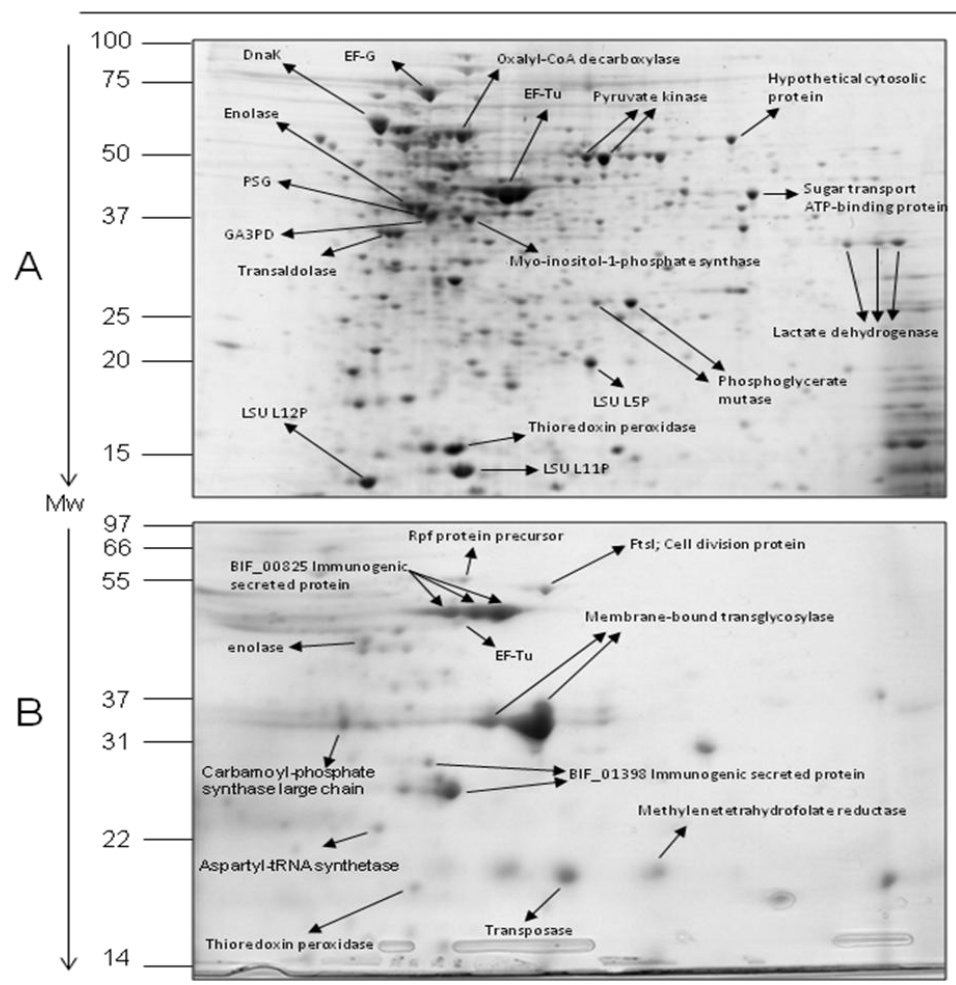


Figure 4.2. **Gel images of the inctracellular (A) and the extracellular fraction (B) protein fractions of *Bifidobacterium animalis* subsp. *lactis* BB-12. Iso-electric point and molecular weight markers are designated, as well as spots corresponding to some of the most abundant proteins. PSG: Phosphoglycerate kinase; GA3PD: Glyceraldehyde-3-phosphate dehydrogenase.**

may be related to the non-cytoplasmic localization of some of the proteins involved in the uptake and transport of nucleotides. The relatively high number of proteins that were not assigned to any. category, or whose functional annotation was classified as general or hypothetical (25 proteins, 30.4% of the unique proteins identified) may be explained by the fact that many of the proteins secreted into the medium or bound at the cell surface are poorly characterised.

In a study that compared proteins expressed by *B. longum* NCC2705 under *in vivo* growth in a rabbit intestinal simulator with those expressed during growth *in vitro*, 19 up-regulated proteins were identified (Yuan *et al.* 2008). In the current study, homologs from BB-12 (50-93% sequence

Table 4.2. Unique proteins of BB-12 identified by MALDI TOF MS from the spots of the gel shown in Fig. 4.1. For more detailed description of the protein hits (including accession numbers) see Table A.3. (Appendix).

Locus	GB acc. #Protein ID	Com*	Structural features†	COG¶	Identified *B. longum* homolog‡	Homologous protein identified in earlier studies‡
BIF_00045	Protein Translation Elongation Factor G (EF-G)	Cyt		J	BL1098	IV, CE, ME
BIF_00066	Alkanesulfonates transport ATP-binding protein	Mem		P		
BIF_00083	ABC transporter ATP-binding protein	Mem		P		
BIF_00116	Type I restriction-modification system DNA	Cyt		S		
BIF_00120	Trigger factor, ppiase	Cyt		O	BL0947	IV, CE
BIF_00129	GTP-binding protein era	Mem		R		
BIF_00203	DNA polymerase III, delta' subunit	Cyt		L		
BIF_00207	Bile salt hydrolase	Ukn		M	BL0796	IV, CE, ME
BIF_00212	Sugar-binding protein	Ukn	NCS	G	BL1163	EX
BIF_00213	LSU ribosomal protein L5P	Cyt	NCS	J	BL1592	CE, ME
BIF_00277	Phosphoglycerate kinase	Cyt		G	BL0707	IV, CE
BIF_00278	Hypothetical cytosolic protein	Cyt		S		
BIF_00293	Dipeptide transport ATP-binding protein; DppF	Mem		R	BL1348	ME
BIF_00315	Division specific D,D-transpeptidase	Mem	1 (NTA)	M	BL1317	EX
BIF_00317	Methylenetetrahydrofolate reductase	Cyt	SP, NCS	E		
BIF_00325	Adenylate kinase	Cyt		F		
BIF_00348	Glutamine synthetase	Cyt		E	BL1076	IV, CE, ME
BIF_00364	Transcriptional regulator, LysR family	Cyt		K		
BIF_00377	Probable glutamate-ammonia-ligase	Cyt		O, T		
BIF_00400	60 kDa chaperonin GROEL	Cyt		O	BL0002	IV, CE
BIF_00414	Hypothetical cytosolic protein	Cyt		R		
BIF_00469	Maltose/maltodextrin-binding protein	Ukn		G		
BIF_00480	Hypothetical protein	Cyt	1 (NTA), NCS	None		
BIF_00497	Peroxiredoxin	Cyt	NCS	O	BL0615	ME
BIF_00539	ATP-binding protein (contains P-loop)	Cyt		R		
BIF_00592	Oxalyl-CoA decarboxylase	Cyt		H, E	BL0296	ME
BIF_00608	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate	Cyt		I		
BIF_00644	Thioredoxin peroxidase	Cyt		O		
BIF_00678	UDP-*N*-acetylenolpyruvoylglucosamine reductase	Cyt		M		
BIF_00679	Inosine-uridine preferring nucleoside hydrolases	Cyt		F		
BIF_00682	Arginine repressor	Cyt		K		
BIF_00737	Oligopeptide-binding protein	Ukn		E	BL1386	EX, ME
BIF_00758	Hypothetical exported protein	Ukn	SP, 1 NTA), NCS	F		

BIF_00784	Hypothetical cytosolic protei	Ukn	NCS	S		
BIF_00823	Cell division ATP-binding protein	Mem	NCS	D		
BIF_00825	Immunogenic secreted protein	Ex	1 (NTA), NCS	R	BL1181	EX
BIF_00860	Diaminopimelate epimerase	Cyt	2 (NTA, CTA), NCS	E		
BIF_00888	*O*-Acetyltransferase (cell wall biosynthesis)	Cyt		R		
BIF_00936	Two component system histidine kinase	Mem	1 (NTA)	T		
BIF_00963	Transketolase	Cyt		G	BL0716	IV, CE
BIF_00973	Peptidyl-prolyl cis-trans isomerase	Cyt		O	BL0354	ME
BIF_00998	Collagen adhesion protein	Cw	2 (NTA, CTA), SP, NCS, SRM	None		
BIF_00999	Hypothetical protein	Ukn	2 (NTA, CTA), SRM	None		
BIF_01012	23S rRNA methyltransferase	Cyt	SP, NCS	J		
BIF_01067	hypothetical signal transduction protein	Cyt		T		
BIF_01084	Tetrapyrrole (Corrin/Porphyrin) methylase family protein	Cyt		R		
BIF_01087	Manganese-binding protein	Mem	1 (NTA)	P		
BIF_01115	Inorganic pyrophosphatase	Cyt	NCS	C		
BIF_01132	Glycerol dehydrogenase	Cyt	NCS	C		
BIF_01172	Thioredoxin	Cyt		O		
BIF_01173	Hypothetical protein	Ukn	1 (NTA)	None	BL0352	IV, ME
BIF_01178	Fibronectin-binding protein	Ukn	2 (NTA, CTA), SRM	None		
BIF_01197	Enolase	Cyt	NCS	G	BL1022	CE, ME
BIF_01234	Manganese-binding protein	Mem	1 (NTA)	P		
BIF_01290	Membrane-bound transglycosylase	Ukn	NCS	S		
BIF_01318	ClpB	Cyt	NCS	O		
BIF_01349	Ribonucleoside-diphosphate reductase α chain	Cyt		F		
BIF_01353	Formate-tetrahydrofolate ligase	Cyt		F	BL0478	CE, ME
BIF_01394	hypothetical protein	Ukn		S		
BIF_01396	Ketol-acid reductoisomerase	Cyt		H	BL0530	CE, ME
BIF_01398	Immunogenic secreted protein	Ex		R		
BIF_01486	Arginyl-tRNA synthetase	Cyt	NCS	J		
BIF_01492	Glycosyltransferase	Cyt		M		
BIF_01521	α-1,3-Galactosyltransferase	Cyt		M		
BIF_01523	UDP-glucose 6-dehydrogenase	Cyt		M		
BIF_01563	Monosaccharide translocase (flippase type)	Mem	4	S	BL0476	ME
BIF_01565	Hypothetical protein ylxX/ylxW	Ukn	NCS	S		
BIF_01597	Oligo-1,6-glucosidase	Cyt	NCS	G		
BIF_01614	DnaK	Ukn	NCS	O	BL0520	CE
BIF_01620	Maltose/maltodextrin-binding protein	Ukn	NCS	G		
BIF_01641	UDP-galactofuranosyltransferase	Cyt	NCS	R		
BIF_01657	Iron-binding ferritin-like antioxidant protein	Cyt		P		

BIF_01677	Rpf protein precursor	Cw	1 (NTA)	S	BL0658	EX
BIF_01746	Peptidyl-prolyl cis-trans isomerase	Cyt	NCS	O		
BIF_01863	Hypothetical protein	Cyt		S		
BIF_01914	Cell wall-associated hydrolase	Mem	1 (NTA)	M	BL1663	EX
BIF_01915	Uridylate kinase	Cyt	NCS	F	BL1505	ME
BIF_01972	Protein Translation Elongation Factor Tu	Cyt		J	BL1097	CE, ME
BIF_02023	Transposase	Cyt		L		
BIF_02033	Hypothetical protein	Cyt		S		
BIF_02061	Hypothetical protein	Cyt		None		
BIF_02065	Aspartyl-tRNA synthetase	Cyt		J	BL0018	CE, ME

[*] Cellular localisation of proteins was predicted using Psortb. Cyt: cytoplasmic; Mem: membrane; Ukn: unknown; Ex: extracellular; Cw: cell wall. [†] predictions of structural features: SP, signal peptide; NTA and CTA: N- and C-terminal membrane anchor, respectively; NCS: non-classical secretions; SRM: sortase recognition motif. Numbers designate the number of predicted trans-membrane domain. [¶] COG functional classification: For the letters category key see Fig. 4.3 or http://www.ncbi.nlm.nih.gov/COG/. [‡] Homologous proteins from *B. longum* (according to the genome sequence of the NCC2705 strain) that were up-regulated during *in vivo* growth (IV) in a rabbit intestinal colon model compared to *in vitro* growth (Yuan *et al.* 2008), or identified in cell envelope (CE), membrane (ME) or extracellular (EX) fractions (Ruiz *et al.* 2009; Sánchez et al. 2008b).

identity) were identified for 10 of these proteins (designated with "IV" in Table 7.3). Six of these proteins – choloylglycine hydrolases (BIF_00207), phosphoglycerate kinase (BIF_00277), factor Tu (EF-TU; BIF_01972), glutamine synthetase (BIF_00348), 60 kDa chaperonin GROEL (BIF_00400) and transketolase (BIF_00963) – were ascribed moonlighting functions associated with host interactions (discussed in sections 4.3.3.1-2). The remaining proteins were oligopeptide-binding protein (BIF_00737), protein translation elongation factor G (BIF_00045) trigger factor, ppiase (BIF_00120), and a hypothetical protein (BIF_01173). With the exception of BIF_00737 and BIF_01173, the remaining 8 proteins have a cytoplasmic function. The *in vivo* up-regulation of these proteins, which are postulated to play a central role in the adaptation of the bacterium to the GIT environment, may provide the biological rationale for their presence in the extracellular fractions of BB-12, as secreted proteins are those expected to come in contact with the host intestinal cells.

4.3.3. Proteins with predicted non-cytoplasmic functions

According to sequence homology-based predictions, 31 of the proteins identified in the present study exert their function outside the bacterial cell or at its surface (Tables 4.2–4.5). These proteins are roughly divided into three main groups: solute-binding proteins, cell wall-metabolizing proteins, and proteins with putative host interacting roles. A few proteins were assigned to more than a single group; *i.e.* the two manganese-binding proteins (BIF_01087 and BIF_01234) and the three cell

wall-metabolizing proteins (BIF_01523, BIF_01677 and BIF_01914), which may also be involved in host-interactions (Fig, 4.4).

4.3.3.1. Solute-binding proteins

Four SBPs were identified in the extracellular fractions of BB-12 (Table 4.3), *i.e.* two pairs of maltose/maltodextrin-binding proteins (BIF_00469 and BIF_01620) and manganese-binding proteins (BIF_01087 and BIF_01234), respectively. Two additional SBPs, a sugar-binding protein (BIF_00212) and an oligopeptide-binding protein (BIF_00737) were identified in a similar study of BB-12 cultures grown on xylo-oligosaccharides (XOS) as carbon source (unpublished data). In accordance with the identification of SBPs in the present study, 15 and three SBPs were previously identified from the extracellular and cell envelope proteomes of *B. longum* NCIMB 8809 (Sánchez *et al*. 2008_b; Ruiz *et al*. 2009), respectively. Of special notice is the identification of BIF_00212, whose abundance increased in the intracellular fraction of BB-12 grown on XOS compared to glucose, a finding verified by transcriptional analyses (Gilad *et al*. 2010). According to the model for XOS catabolism suggested in the latter study, binding of XOS by BIF_00212 at the cell surface is the first step in XOS utilization. The fact that BIF_00212 was found in XOS grown culture supernatants supports this model.

The presence of two manganese-binding proteins in the extracellular fraction of BB-12 is in agreement with the pivotal role that manganese homeostasis plays in a variety of bacterial cellular processes, including sensory mechanisms, virulence and adhesion [16]. In addition, these proteins may be involved in adhesion to host cells, as inferred by homology to adhesive streptococcal proteins (Table 4.5).

Another identified protein, oligo-1,6-glucosidase (EC 3.2.1.10; BIF_01597), may be involved in utilization of oligosaccharides. This enzyme belongs to the α-amylase family (known also as the glycoside hydrolases family 13, which has the ability to hydrolyse starch and its degradation products The secretion of this enzyme may not only enhance the nutrient uptake capacity of the bacterium, but also have implications for the generation of short chain fatty acids that are shown to have a beneficial effect on the human intestinal epithelium (Sánchez *et al*. 2008_a). BIF_01597, and the oligosaccharide-binding proteins identified in the present study are assumed to contribute to the survival and adaptation of the bacterium to the GIT through the degradation and uptake of oligosaccharides, which are common carbon-sources in this milieu.

The presence of two manganese-binding proteins in the extracellular fraction of BB-12 is in agreement with the pivotal role that manganese homeostasis play in a handful of bacterial cellular

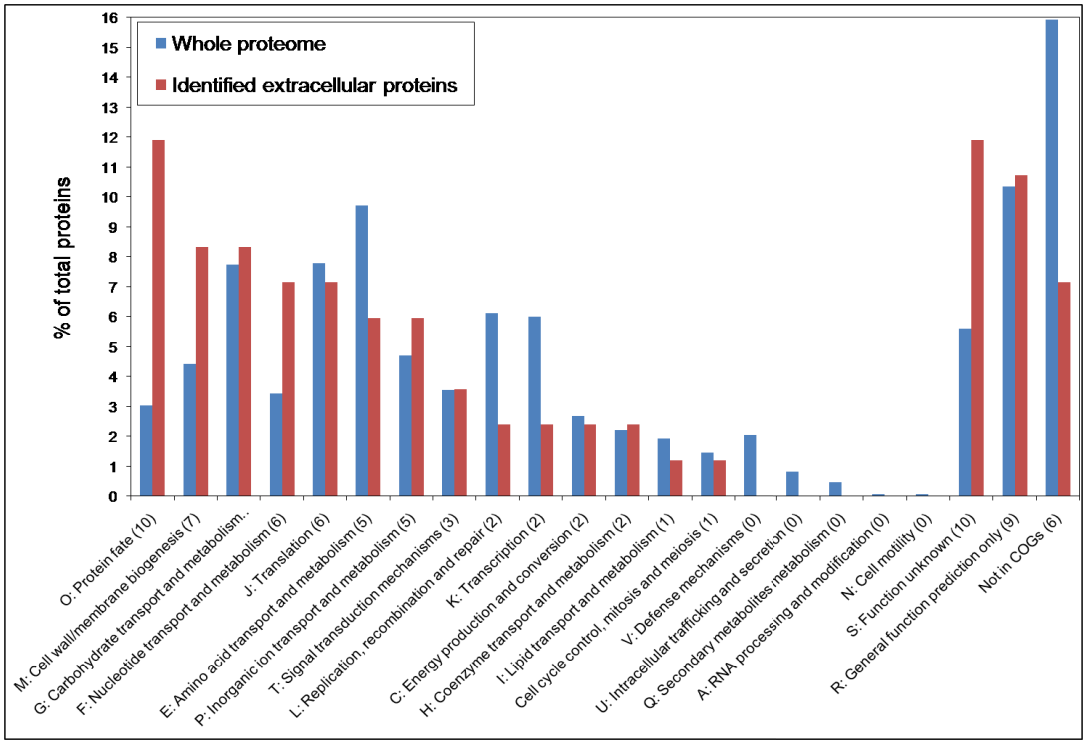


Figure 4.3. **COG functional classification of the proteins identified in the extracellular fraction of *Bifidobacterium animalis* subsp. *lactis* BB-12, compared with the total proteome. Values in parentheses designated at the end of each functional category represent the number of proteins assigned to this category, which were identified in this study.**

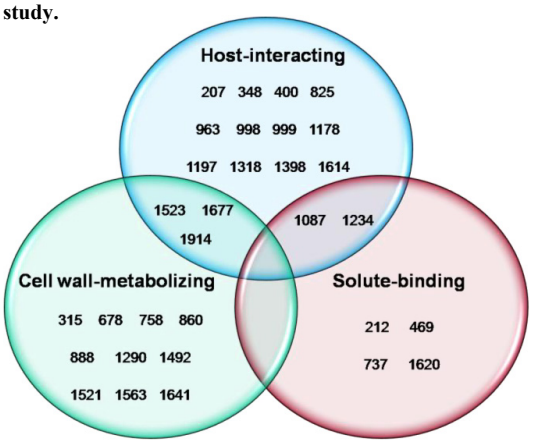


Figure 4.4. **Venn diagram describing the distribution of the proteins identified in this study which are predicted to have an non-cytoplasmic function within the designated functional groups. Numbers refer to the *Bifidobacterium animalis* subsp. *lactis* BB-12 locus tags (as annotated in GenBank accession num. CP001853), where the BIF_0 prefix was removed.**

.**Table 4.3. Solute-binding proteins identified in the extracellular fraction of BB-12.**

Locus	Protein ID	Homologous protein	Acc. num.	% seq. ID	Experimentally-documented fnunction	Organism	Reference
BIF_00212	Sugar-binding protein	maltose/malto-dextrin-binding protein	AAZ51685	25	critical for maltose utilization and effects colonization of mouse ropharynx	*Streptococcus pyogenes* MGAS5005	Shelburne *et al.* 2006
BIF_00469	Maltose/malto-dextrin-binding protein	Maltose-binding periplasmic protein	P0AEX9	21	binding linear malto-oligosaccha-rides and maltodextrins	*E. coli* K-12	Quiocho *et al*. 1997
BIF_00737	Oligopeptide-binding protein	dipeptide-bind-ing protein dppE	P26906	27	essentiel for di-peptide transport	*Bacillus subtilis*	Mathiopoulos *et al*. 2006
BIF_01087	Manganese-binding protein	Periplasmic chelated iron-binding protein yfeA	Q56952	23	transporting iron and manganese ions	Yersinia pestis	Bearden and Perry 2002
BIF_01234	Manganese-binding protein	Zinc-binding lipoprotein AdcA	O05703	24	Encoded by a gene that is a part from an operon vital for transport of Mn2+ and Zn2+ ions	Streptococcus pneumonia	Dintilhac et al. 1997
BIF_01620	Maltose/malto-dextrin-binding protein	maltose/malto-dextrin-binding protein	AAN59213	28	Involved in transport of malto-dextrins (up to 7 glucose units)	*Streptococcus mutans* UA159	Webb *et al*. 2008

mechanisms, including sensory mechanisms, virulence and adhesion (Kehres and Maguire 2003). These proteins are also homologous to adhesive streptococcal proteins (section 4.3.3.3.2.).

4.3.3.2. Cell wall-metabolising proteins

In the present study, 11 proteins whose function is related to cell wall biogenesis or modification were identified (Table 4.3). Among these proteins, 5 were annotated as transferases – four glycosyltransferases (BIF_00315, BIF_01290, BIF_01492 and BIF_01641) and a single acetyltransferase (BIF_00888).

The identified cell wall-metabolizing proteins also include two proteins containing a transpeptidase domain (division specific D,D-transpeptidase [BIF_00315] and hypothetical exported protein [BIF_00758]), an epimerase (BIF_00860), and two enzymes involved in reduction of intermediates of cell wall building blocks (UDP-*N*-acetylenolpyruvylglucosamine reductase [BIF_00678] and UDP-glucose 6-dehydrogenase [BIF_01523]). The product of the reaction catalyzed by BIF_01523, UDP-glucuronic acid, is an important component of antiphagocytic capsular structures composed of exo- and lipopolysaccharide (EPS and LPS, respectively). Deletion of the gene encoding a protein from the Gram-negative pathogen *Proteus mirabilis* homologous to

BIF_01523 decreased the expression of virulence factors and the ability of the bacterium to invade human urothelial NTUB1 cells [17]. This finding, along with the up-regulation of the gene coding for BIF_01523 under incubation of BB-12 with Caco-2 cells (Valina *et al.*, submitted for publication) implies that exposure to host intestinal cells may induce production of EPS and LPS that assist in adhesion and colonization.

Two additional proteins that may play a role in peptidoglycan (PG) modification or autolysis that were identified in the present study are the cell wall-associated hydrolase (BIF_01914) and Rpf (resuscitation promoting factor) protein precursor (BIF_01677). These proteins may also be associated with adhesion to host intestinal cells (section 4.3.3.3.2).

4.3.3.3. Host interacting proteins

Based on experimental evidence from studies of similar proteins in (mostly) Gram-positive bacteria, 18 of the identified proteins may play a role in interactions with human intestinal cells or with constituents of the extracellular matrix (ECM) of the GIT (Table 4.5). These interactions involve the following features: (i) binding to plasminogen (plg); (ii) adhesion to intestinal cells or proteins of the ECM; (iii) elicitation of immunogenic response.

4.3.3.3.1 Plasminogen-binding proteins

Plasminogen is the proenzyme of plasmin, a serine protease that plays an important role in fibrinolysis, homeostasis and degradation of ECM proteins [18]. Due to its versatile proteolytic features and high abundance in human plasma and extracellular fluids, plg is commonly bound to the cell surface of pathogenic bacteria (Li et al. 2011). The subsequent conversion of plg to active plasmin

supplies these bacteria with a variety of proteolytic activities that facilitate their movement across physical and molecular barriers during colonization (Lahteenmaki *et al*. 2005).

Investigation of the binding of bifidobacterial strains demonstrated that five *Bifidobacterium animalis* subsp. *lactis* BI-07 proteins bind to human plg at the cell surface (Candela *et al*. 2007) and four of these – choloylglycine hydrolase (BIF_00207), glutamine synthetase (BIF_00348), enolase (BIF_01197) and DnaK (BIF_01614) – were identified in the present study (Table 2). Based on the high homology (87–100% DNA sequence identity) within the *B. animalis* subsp. *lactis* taxon, and the fact that another *B. animalis* subsp. *lactis* strain (DSM 10140) also binds plg (Candela *et al*. 2007), it is hypothesized that these proteins may promote the colonization of BB-12 in the GIT *via* capture of human plg.

Table 4.4. Proteins identified in the extracellular fraction of BB-12 that may possess cell wall-metabolising functions.

Locus	Protein ID	homologous protein	acc. #	% seq. ID	Experimentally-documented fnunction	Organism	Reference
BIF_00315	Division specific D,D-transpeptidase	transpeptidase involved in septal peptidoglycan synthesis (penicillin-binding protein 3)	AAC73195	30	Peptidoglycan transglycosylase activity	*E. coli* K-12 substr. MG1655	Uehara and Park 2008
BIF_00678	UDP-*N*-acetylenolpy-ruvylglucos-amine reductase	n.i.			reducing UDP-*N*-acetylglucosamine enolpyruvate with the concomitant oxidation of NADPH	*S. pneumonia*	Sylvester *et al.* 2001
BIF_00758	Hypothetical exported protein	n.i.			L,D-transpeptidase that gives rise to an alternative pathway for peptidoglycan cross-linking	*Enterococcus faecium*	Biarrotte-Sorin *et al*, 2006
BIF_00860	Diaminopime-late epimerase	Diaminopimelate epimerase (dapF)	AAC22409	29	amino acid racemase, inverting the configuration at the α-carbon of aPG diaminopimelic acid	*Haemophilus influenzae*	Lloyd *et al.* 2004
BIF_00888	*O*-acetyltransfe-rase (cell wall biosynthesis)	Maltose *O*-acetyltransferase	NP_414992	46	acetyl CoA-dependent acetylation of the 6-hydroxyl group of their respective sugar substrates.	*E. coli* K-12 substr. MG1655	Lo Leggio *et al.* 2003
BIF_01290	Membrane-bound trans-glycosylase	n.i.			cleaving β-1,4-glycosidic bond between *N*-acetyl-muramic acid and *N*-acetyl-D-glucosamine	*S. pneumonia*	Bateman *et al.* 2005
		n.i.			possess a lysozyme-like domain thought to be involved in cell wall modification through the hydrolysis of the glycan backbone of peptidoglycan	*Mycobacterium tuberculosis* H37Rv	Cohen-Gonsaud *et al.* 2004
BIF_01492	Glycosyl-transferase	Amylovoran biosynthesis glycosyltrans-ferase AmsE	Q46635	31	involved in amylovora EPS biosynthesis	*Erwinia amylovora*	Bernhard *et al.* 1993
BIF_01523	UDP-glucose 6-dehydro-genase	UDP-glucose 6-dehydrogenase	AAK34829	53	biosynthesis of UDP-glucuronic acid, an important building block of antiphagocytic EPS and LPS capsular structures	*Streptococcus pyogenes* M1 GAS	Campbell *et al*. 1997

BIF_01641	UDP-galacto-furanosyltrans-ferase	bifunctional UDP-galactofuranosyl transferase GLFT	CAA17872	25	transfers Galf to a range of both β-(1,5) and β-(1,5) linked digalacto-furanosyl neoglycolipid acceptors	*M. tuberculosis* H37Rv	Alderwick *et al.* 2008
BIF_01677	Rpf protein precursor	resuscitation-promoting factor rpfB	CAB08136	23	possess a lysozyme-like domain thought to be involved in cell wall modification through the hydrolysis of the glycan backbone of peptidoglycan	*M. tuberculosis* H37Rv	Cohen-Gonsaud *et al.* 2004
BIF_01914	Cell wall-associated hydrolase	invasion-associated protein p60	CAC98661	40	involved in cell division and actin-based motility	*Listeria monocytogenes* EGD-e	Pilgrim *et al.* 2003

n.i.: not identified

4.3.3.3.2. Adhesion-promoting proteins

Adhesion of bacteria to human intestinal cells or ECM proteins is a vital step for transient intestinal colonization. Bifidobacteria have been shown not only to adhere to a variety of epithelial cells *in vitro*, but also to inhibit adhesion of pathogenic enterobacteria (Liu *et al*. 2010). Homologs to 9 of the proteins identified in the extracellular proteome of BB-12 have been described to be involved in adhesion to human intestinal cells or to different components of the ECM (Table 2)

Table 4.5. Proteins identified in the extracellular proteome of *Bifidobacterium animalis* subsp. *lactis* BB-12 that may possess host-interacting functions.

Protein ID	Mascot PMF Score[a]	Extracellular function	Homologous protein (Acc. Number)	% seq' ID	E value	Organism	Reference
Plasminogen-binding proteins							
Choloylglycine hydrolase	119	- binding to human plasminogen	Bile salt hydrolase (ACS47792)	100	0	*Bifidobacterium animalis* subsp. *lactis* DSM10140	(Candela *et al.* 2007)
Glutamine synthetase	48		Glutamine synthetase 1 (ACS47638)	100	0	*Bifidobacterium animalis* subsp. *lactis* DSM10140	
Enolase	193		α-Enolase (AAZ22544)	87	0	*Bifidobacterium animalis* subsp. *lactis* BI-07	
DnaK	100		DnaK (AAV84834)	100	0	*Bifidobacterium animalis* subsp. *lactis* DSM10140	
Adhesion-promoting proteins							
60 kDa chaperonin GROEL	84	- attaching to human mucus - enhancing secretion of IL-8 from HT29 cells - triggering aggregation of *Helicobacter pylori*	60 kDa chaperonin (Q9KJ23)	58	$< e^{-50}$	*Lactobacillus johnsonii* NCC 533	(Bergonzelli *et al.* 2006)
Collagen adhesion protein	117	- binding to type I collagen	Collagen adhesion protein (YP_059477)	24	$7e^{-11}$	*Streptococcus pyogenes* MGAS1039	(Kreikemeyer *et al.* 2005)
Hypothetical protein	72	- adhering to carbohydrate or peptide structures of the host	Fimbrial structural subunit (AAC13545)	24	$9e^{-05}$	*Actinomyces naeslundii*	(Yeung 1990)
Manganese-binding protein (BIF_01087)	85	- laminin attachment-mediated adhesion to epithelial cells	Putative laminin-binding protein (NP_269968)	21	$2e^{-10}$	*S. pyogenes* M1 GAS SF370	(Terao *et al.* 2002)
Manganese-binding protein (BIF_01234)	61			22	$2e^{-12}$		
Manganese-binding protein (BIF_01087)	91	- involvement in virulence in intranasal and intraperitoneal challenge of mice and adherence to type II pneumocytes	ABC transporter, substrate binding lipoprotein (YP_816919)	24	$8e^{-11}$	*Streptococcus pneumonia* D39	(Berry *et al.* 1996)
Manganese-binding protein (BIF_01234)	71			23	$1e^{-09}$		
Fibronectin-binding protein	148	- attaching to a pharyngeal cell line and generation of biofilm in human cells	Pilin (Q9A1S2)	33	$7e^{-08}$	*S. pyogenes* M1	(Manetti *et al.* 2007)

Protein translation elongation factor Tu	147	- attaching to human mucus - enhancing secretion of IL-8 from macrophages and HT29 cells	EF-Tu (NP_964865)	58	$<e^{-50}$	*Lactobacillus johnsonii* NCC 533	(Granato *et al.* 2004)
		- binding to human fibronectin	EF-Tu (NP_110354)	63	$<e^{-50}$	*Mycoplasma pneumoniae* M129	(Dallo *et al.* 2002)
UDP-glucose 6-dehydrogenase	50	- upregulation of the expression of the corresponding gene upon incubation with Caco-2 cells	UDP-glucose 6-dehydrogenase (ADC84776)	100	0	*Bifidobacterium animalis* subsp. *lactis* BB-12	Valina *et al.*, submitted for publication
Oligo-1,6-glucosidase	60	- adhesion to porcine epithelium and mucus *in vitro*	Surface-anchored amylopullulanase (YP_003027676)	40	$8e^{-14}$	*Streptococcus suis* P1/7	(Ferrando *et al.* 2010)
Immunogenic proteins							
Immunogenic secreted protein (BIF_00825)	87	- binding to antibodies in human serum	Immunogenic secreted protein precursor-like protein (NP_665366)	42	$2e^{-24}$	*Streptococcus pyogenes* MGAS315	(Lei *et al.* 2000)
Immunogenic secreted protein (BIF_01398)	104	- binding to antibodies in human serum	Immunogenic secreted protein precursor-like protein (NP_665366)	46	$4e^{-28}$	*S. pyogenes* MGAS315	(Lei *et al.* 2000)
Immunogenic secreted protein (BIF_00825)	87	- stimulating the activation of Akt in IECs and inhibition of TNF-induced IEC apoptosis	Surface antigen (CAR85926)	32	$6e^{-14}$	*Lactobacillus rhamnosus* GG	(Yan *et al.* 2007)
Immunogenic secreted protein (BIF_01398)	104	- stimulating the activation of Akt in IECs and inhibition of TNF-induced IEC apoptosis	Surface antigen (CAR85926)	36	$1e^{-12}$	*L. rhamnosus* GG	(Yan *et al.* 2007)
Transketolase	67	- binding to goat anti-rabbit Immunoglobulin G antibody	Transketolase (YP_040758)	40	$<e^{-50}$	*Staphylococcus aureus* MRSA-M2	(Brady *et al.* 2006)
ClpB	67	- exerting immunological activity during host–pathogen interaction	ClpB (EAQ73285)	48	0	*Campylobacter jejuni*	(Thies *et al.* 1999)
Rpf protein precursor	152	- binding to rat anti-mouse immunoglobulin G antibodies - enhancing proliferation of specific mice lymph node T-cells - triggering cytokine response	rpfB (CAB08136)	23	$6e^{-11}$	*Mycobacterium tuberculosis* H37Rv	(Yeremeev *et al.* 2003)
Cell wall-associated hydrolase	75	- stimulating the activation of Akt in IECs - inhibiting TNF-induced IEC apoptosis	NLP/P60 protein (YP_003170070)	34	$8e^{-14}$	*L. rhamnosus* GG	(Yan *et al.* 2007)

[a] Peptide mass fingerprinting (PMF) score obtained using the database search algorithm Mascot (http://www.matrixscience.com) with the genome sequence of *Bifidobacterium animalis* subsp. *lactis* BB-12 as taxonomy entry. Scores > 45 are significant.

Collagen is one of the major proteins of the ECM and is susceptible to invasion of pathogenic Gram-positive and -negative bacteria under injury (Shen and Ljung 1993). Adhesion of probiotic bacteria to collagen is predicted to interfere with colonization and invasion of pathogenic bacteria. A few bifidobacterial strains, including BB-12, have been shown to bind different types of collagen, though in relatively low rates (Ouwehand *et al*. 2004) . The protein BIF_00998 shares homology with the collagen adhesion protein from *Streptococcus pyogenes* MGAS10394, and with a few other cell surface adhesion-related proteins from *S. pyogenes* strains. The fact that the recombinant MGAS10394 protein was described to attach to type I and type IV collagen (Kreikemeyer *et al*. 2005) suggests that BIF_00998 also possesses collagen binding capacity.

Bacterial fimbriae are long fibrous structures composed of pilus subunits, which serve as adhesive anchors that facilitate attachment to host cells and have been associated with biofilm formation (Proft *et al*. 2009). The assembly of fimbriae is catalyzed by sortase, a membrane-anchored transpeptidase that recognizes a sequence motif in the pilus subunits, binds them together *via* intermolecular isopeptide bonds and covalently anchors the fimbriae oligomer into the cell wall PG (Ton-That *et al*. 1999). Fibrous structures were observed on the surface of *Lactobacillus rhamnosus GG* (LGG), whose binding to human mucus was found to depend strongly on the expression of one of the pilus subunits (Kankainen *et al*. 2009). Preliminary electron micrographs revealed fimbriae-like structures also on the surface of *B. longum* (Schell *et al*. 2002). .

In the present study, two proteins (BIF_00998 and BIF_00999) that possess a C-terminal sortase recognition motif (Fig. 4.5) were identified from a protein spot with an $M_r > 200$ (Fig. 4.1, spot #1 & Table A.2). The sequence coverage of the protein hit corresponding to BIF_00998 is 11%).

Figure 4.5. Schematic illustration of the organisation of the genes coding for proteins predicted to be involved in fimbriae formation in *Bifidobacterium animalis* subp. *lactis* BB-12. Fimbrial structures may be produced by generation of oligomeric structures of fimbriae subunits containing a sortase recognition motif – hypothetical protein (BIF_00999) and collagen adhesion protein (BIF_00998). A sortase protein (BIF_01042) is predicted to cleave these proteins at their recognition site, bind them together through intermolecular isopeptide bonds and covalently anchor the resulting fimbriae oligomer into the cell wall peptidoglycan. The function of BIF_02156 is unclear.

Moreover, the genes coding for these proteins are separated by a single ORF of 120 nucleotides, and a gene encoding a sortase (BIF_01042) is located immediately upstream of the gene coding for BIF_00998 (Fig. 4.5). All three proteins are homologous to proteins documented to play a role in fimbriae formation and and/or collagen-binding in Gram-positive bacteria (Table 4.5), and the positioning of these genes resembles the fibronectin-binding, collagen-binding, T antigen (FCT) genomic region identified in *Streptococcus pyogenes*, whose gene products were reported to be associated with human infection (Kratovac *et al*. 2007). In light of these observations, it can be postulated that BIF_00998 and BIF_00999 are substrates for the sortase and are involved in fimbriae formation and subsequent adhesion to host proteins like collagen and fibronectin, another important ECM protein. Another protein potentially involved in fibronectin binding and/or fimbriae formation is the fibronectin-binding protein BIF_01178 that contains a repeated conserved domain from *S. pyogenes* M1 GAS. This domain is characteristic of adhesion and signaling (Whittaker *et al*. 2002), biofilm formation, and attachment to human pharyngeal cell lines (Manetti *et al*. 2007).

Finally, three additional proteins predicted to play a role in adhesion are the oligo-1,6-glucosidase (BIF_01597) and the chaperones GROEL (also called 60 kDa chaperonin) and EF-TU, the two latter are regarded as moonlighting proteins. An amylopullulanase from *Streptococcus suis* P1/7 that shares sequence similarity with BIF_01597 promoted adhesion to porcine epithelium and mucus *in vitro* (Ferrando *et al*. 2010). The GROEL and EF-TU homologs from *Lactobacillus johnsonii* NCC 533 were reported to attach to human mucus (Granato *et al*. 2004) and the 60 kDa chaperonin triggered aggregation of the pathogen *Helicobacter pylori*. EF-TU from *Mycoplasma pneumoniae* M129, which is also homologous to the BB-12 protein, was reported to bind human fibronectin [34]. It can be hypothesized that the GROEL and EF-TU of BB-12 demonstrate similar adhesive and immunogenic properties.

4.3.3.3.3. Immunogenic proteins

One of the central claims attributed to probiotic bacteria is their ability to modulate the immune response of the host through the interaction with important elements in the maintenance of gut homeostasis like intestinal epithelial cells (IECs) and dendritic cells (DCs). Immunomodulating effects were observed in a variety of bifidobacterial strains in general and in BB-12 in particular [35–36]. Administration of the strain was shown to alleviate the inflammatory response in eczema patients [37] and to enhance phagocytosis of *Escherichia coli* sp. by leukocytes isolated from the blood [38]. Another study demonstrated that individuals who received BB-12 experienced a decrease in the level of tumor necrosis factor (TNF)-α, as well as a reduction of interleukin-10 (IL-

10), which are pro- and anti-inflammatory cytokines, respectively. The biological mechanism behind the decrease in the concentration of IL-10 has yet to be deciphered [35].

In the current study, 6 proteins with potential immunogenic properties were identified (Table 4.5). The chaperonic protease ClpB shares 47% sequence identity with the corresponding protein of *Campylobacter jejuni*, which triggers the immune system during host-pathogen interactions [39]. The Rpf protein precursor from BB-12 (BIF_01677) possesses a G5 protein family domain, which is found in a wide range of extracellular peptidases that cleave human IgA [40]. Accordingly, immunogenic properties were documented in Rpf-like proteins from *Mycobacterium tuberculosis* H37Rv with sequence similarity to BIF_01677 [41]. These proteins were also suggested to be associated with cell wall adhesion and biofilm formation [42].

The surface antigen protein and the cell wall-associated glycoside hydrolase (NLP/P60 protein) of *Lactobacillus rhamnosus* GG (LGG), known as p40 and p75, respectively, stimulate the activation of Akt (protein kinase B protein family) in IECs and inhibit TNF-induced IEC apoptosis [43]. Sequence alignments revealed that p40 is similar to regions near the C-terminus of the immunogenic secreted proteins BIF_00825 and BIF_01398 identified in the present study. Similar segments of BIF_00825 and BIF_01398 are homologous with the immunogenic secreted protein precursor-like protein from *S. pyogenes* MGAS315 that was shown to trigger appearance of antibodies in human serum after infection by the bacterium [44]. The cell wall-associated hydrolase (BIF_01914) is homologous with p75 and may thus exhibit immunomodulating features. Finally, the presence of the key glycolytic enzyme transketolase (BIF_00963) in the extracellular protein fraction of BB-12 is probably another example of a moonlighting protein, as seen for *Staphylococcus aureus* MRSA-M2, where it was described to bind goat anti-rabbit IgG antibody [45].

Taken together, these findings imply that BB-12 is secreting immunomodulative proteins that, along with the proteins predicted to facilitate adhesion to host intestinal cells and ECM proteins of the GIT, assist in identification of proteins that may be involved in interactions of the bacterium with the host at the molecular level.

4.4. Conclusions

The present analysis of the extracellular proteome of *Bifidobacterium animalis* subsp. *lactis* BB-12 gave rise to several noteworthy observations. First, the presence of cell wall- or membrane-anchored SBPs was reported, including proteins predicted to bind di- and oligosaccharides at the cell surface, as well as manganese-binding proteins shown to play a central role in a variety of

cellular processes. Second, the identification of a considerable number of cell wall-metabolizing enzymes suggested to be associated with modification of PG that, apart from being essential for cell division, also results in release of PG fragments that may have influence on host intestinal cells. Third, a most remarkable finding in this work is the identification of 18 proteins that possibly play a role in interaction with or adhesion to host intestinal cells, through recruiting of plasminogen, adhering to collagen and mucin or by eliciting immunomodulatory effects.

The present findings can be used to target studies to corroborate the putative functions ascribed to the identified proteins. This for example can be facilitated through the use of binding or immunogenic assays combined with mutations/deletions of the genes coding for these proteins. Deciphering the factors that play a role in bacteria-host interactions is expected to deepen the insight into the molecular mechanisms underlying the beneficial effects exerted by probiotics in general and by bifidobacteria in particular.

4.5. Materials and methods

4.5.1. Experimental design

Extracellular proteins obtained from *B. animalis* subsp. *lactis* BB-12 culture supernatants (three biological replicates, harvested after 8 h of growth, *i.e.* late exponential growth phase at an OD_{600} of 1.3) were analyzed by 2-DE. Each electrophoresis experiment was run in duplicate. Protein spots from a representative 2D gel were excised and subjected to in-gel digestion followed by MALDI-TOF MS. The proteins identified from these spots are presented.

4.5.2 Strain and growth medium

Bifidobacterium animalis subsp. *lactis* BB-12 (BB-12 is a registered trade mark of Chr. Hansen A/S) was cultured at Chr. Hansen A/S, Hørsholm, Denmark. Growth experiments were performed in a reconstituted MRS medium containing 0.05% cysteine-hydrochloride at 37 °C as previously described [8].

4.5.3 Extraction of extracellular proteins and two-dimensional gel electrophoresis

Cell-free samples for proteome analysis were collected by recovering the supernatants of the bacterial cultures by centrifugation (10 min, 4 ˚C, 3,200×*g*) followed by filtration (0.22 μm pore size). To provide a sufficient amount of proteins for 2-DE and to discard salts and bacterial polysaccharides, filtered supernatants (40 ml) were subjected to two steps of chloroform/methanol precipitation – the first with 4:4:1 (v/v) sample:methanol:chloroform and the second with 1:4:1:3

(v/v) sample:methanol:chloroform:water [9]. Precipitates were dissolved in 15 mM Tris, pH 8.5, 8 M urea, and subjected to protein quantification [10]. Prior to 2-DE, carrier ampholytes, CHAPS and DTT were added to final concentrations of 0.2%, 0.4% and 50 mM, respectively, and the volume was adjusted to 200 µl (corresponding to 50 µg protein). IEF (using 11 cm IPG strips, linear pH gradient 4–7, 18 cm [GE Healthcare, Piscataway, NJ]), reduction and alkylation were performed as previously described [8]. Electrophoresis in the second dimension was carried out using Criterion Tris-HCl (12.5% polyacrylamide) precast gels (Bio-Rad) run on a Criterion Dodeca Cell electrophoresis unit (Bio-Rad) at 200 V for 1 h, followed by fixation and staining with colloidal CBB-G250 as previously reported [8].

4.5.4 In-gel digestion and protein identification by mass spectrometry

Protein spots from the 2-D gels were manually excised and subjected to in-gel tryptic digestion as described [11] with a few modifications [12], leaving out the reduction and alkylation steps performed already prior to 2-DE. MS was carried out on an Ultraflex II MALDI-TOF instrument (Bruker Daltonics, Bremen, Germany) as described [8], and the obtained spectra were analyzed using the FlexAnalysis 3.0.96 program (Bruker Daltonics). Peak lists were acquired using the Biotools 3.1 software (Bruker Daltonics), subjected to Tophat baseline substraction, internal calibration using trypsin autolysis products and keratin contaminants, (which were subsequently discarded) and searched using the Mascot database search algorithm (http://www.matrixscience.com) embedded within Biotools. Mascot searches against the genome sequence of BB-12 (GenBank accession no. CP001853, 1612 ORFs, 587905 amino acid residues) [13] as taxonomy entry, as well as against a decoyed version of the same database, were done with carbamidomethylation (Cys) and oxidation (Met) as fixed and variable modifications, respectively, with tolerance of one missed cleavage and a maximum error tolerance of 80 ppm in the MS data. The peak signal-to-noise ratio was set to 6 and was lowered to 3 when the obtained values for PMF score or the sequence coverage were lower than 60 and 16%, respectively. No restrictions with respect to protein mass and p*I* were made. Protein hits with $P < 0.05$, corresponding to score > 45, and with sequence coverage > 15% (when score < 60) were considered significant. In addition, protein hits for which the Mascot database search resulted in a decoy hit with a score > 45 were discarded.

4.5.5 Bioinformatic analyses of the identified proteins

Signal peptide predictions were obtained using SignalP 3.0 server (http://www.cbs.dtu.dk/services/

SignalP/) with Gram-positive bacteria selected as the organism group and a maximal truncation of 70 residues to each sequence. Predictions were considered positive when the presence of a signal peptide was suggested according to both the D mean score of the neural networks based model and the hidden Markov models. The presence of transmembrane section(s) (TMS(s) was predicted using TMHMM version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Prediction of non-classically secreted proteins was performed using SecretomeP 2.0 Server (http://www.cbs.dtu.dk/services/SecretomeP/) with Gram-positive bacteria selected as the organism group. Functional classification of proteins according to Clusters of Orthologous Groups (COGs) was obtained using the KOBAS (KEGG orthology based annotation system) server (http://kobas.cbi.pku.edu.cn/) using default parameters. Protein sequence alignments of the proteins identified by MS were carried out using BLASTP [14] 2.2.23 at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the non-redundant protein sequences (nr) database. Conserved protein domains were detected as a part of the BLASTP searches, and homologies to protein families were detected using Pfam 24.0 (http://pfam.sanger.ac.uk/). Accession numbers listed refer to GenBank identifiers.

Chapter 5. Insights into physiological traits of *Bifidobacterium animalis* subsp. *lactis* BB-12 through membrane proteome analysis

5.1. Summary

Bifidobacterium animalis subsp. *lactis* BB-12 is a widely used probiotic strain associated with avariety of health-promoting traits. There is, however, only limited knowledge available regardingthe membrane proteome and the proteins involved in oligosaccharide transportin BB-12. We applied two enrichment strategies to improve the identification of membraneproteins from BB-12 cultures grown on glucose and on xylo-oligosaccharides, the latter being an emerging prebiotic substrate recently reported to be fermented by BB-12. Our approachencompassed consecutive steps of detergent- and carbonate-treatment in order togenerate inside-out membrane vesicles and to interfere with binding of membraneassociatedproteins to the membrane, respectively. Proteins in the enriched membranefraction and membrane-associated fraction were digested by lysyl endopeptidase and trypsinfollowed by peptide sequencing by LC-ESI-Q-TOF MS/MS. Ninety of a total of 248 identifiedunique proteins were predicted to possess transmembrane segments (TMSs), and 56 ofthese have more than one TMS. Seventy-nine of the identified proteins are annotated to beinvolved in transport of amino acids, oligosaccharides, inorganic ions, nucleotides, phosphateor exopolysaccharides, or to belong to the F1F0-ATP-synthetase complex and the proteintranslocation machinery, respectively.

5.2. Introduction

Bifidobacteria constitute one of the major genera in the microbiota inhabiting the human gastrointestinal tract (GIT) (Ventura *et al.* 2007_b). Due to their health-promoting (*i.e.* probiotic) properties, e.g. host immunomodulation and induction of anti-pathogenic defense mechanisms (Marco *et al.* 2006) bifidobacteria are commonly used both in food applications and as dietary supplements (Masco *et al.* 2005).

Membrane proteins (MP) account for approximately 30% of all predicted proteins in proteomes (von Heijne *et al.* 2007). Due to their key roles in numerous cellular processes, e.g. metabolite transport, inorganic ion homeostasis, expulsion of toxins and antibiotics, energy metabolism, communication with the surrounding environment, and cell wall modification and biogenesis, MP are essential for the survival of bifidobacteria in the GIT. Additionally, MP are involved in bacteria-host interactions and contribute to the molecular basis for health-beneficial effects exerted by probiotic bacteria (Sánchez *et al.* 2008).

The first studies concerning MP from bifidobacteria focusedon antibiotic resistance and bile tolerance, and consistedof biochemical analysis of recombinantly producedtransport proteins, as well as comparative analysis of membraneprotein profiles by using SDS-PAGE (Margolles *et al.* 2006, Margolles *et al.* 2003). Recently, biletolerance was examined in *Bifidobacterium longum* NCIMB 8809 grown in an *in vitro* bile environment by cell-envelopeproteome analysis using 2D-DIGE and SILAC. This resulted inidentification of 218 proteins, of which 141 were present inthe membrane protein fraction, including 78 transportrelatedproteins constituting a large proportion of the transportersannotated in the genome (Ruiz *et al.* 2009).

Mining the genome sequence of *B. longum* NCC2705 for carbohydrate transport systems identified 19 predicted sugaractiveoperons, including 13 ATP-binding cassette (ABC)-typetransport systems − containing putative permeases for lactose,maltose, raffinose, and fructo-oligosaccharides (FOS), and three major-facilitator superfamily (MFS) transporterspredicted to import sucrose, lactose and glucose/galactose (Parche *et al.* 2007). In addition, growth experiments and microarray analysesconfirmed that the various predicted substrates were utilizedby the bacteriumand that all carbohydrate catabolism-relatedgenes were expressed, some being induced upon growth on lactose, raffinose or FOS (Parche *et al.* 2007).

Recently, transcriptomic and proteomic analysis of thewidely used probiotic strain *B. animalis* subsp. *lactis* BB-12 grown on xylo-oligosaccharides (XOS) suggested that XOS uptake is facilitated via an ABC transport system whose constituentsare induced by XOS (Gilad *et al.* 2010). To increase the coverage of identification of transport proteins in bifidobacteria, membrane fractions prepared from BB-12 grown on either glucoseor XOS were analyzed using a gel-free approach, consistingof lysyl endopeptidase and trypsin digestion of suspended membrane fractions followed by liquid chromatography/electrosprayionization quadrupole time-of-flight-tandem mass spectrometry (LC-ESI-Q-TOF-MS/MS). This first LC–MS/MSbased membrane proteome analysis in bifidobacteria demonstrated the presence of a large number of putative transport systems in BB-12 at the protein level.

5.3. Results and Discussion

5.3.1 Bioinformatic analysis of the identified proteins

A total of 248 proteins were identified in the membrane fractionsby LC–MS/MS and sequence database searching (Supplemental Table A.4) and classified with respect to Subcellular component, Biological processes and Molecular functions based on Gene Ontology (GO) classification (Fig. 5.2). Nearly half of the proteins (132) were assigned to more than one category within the three GO

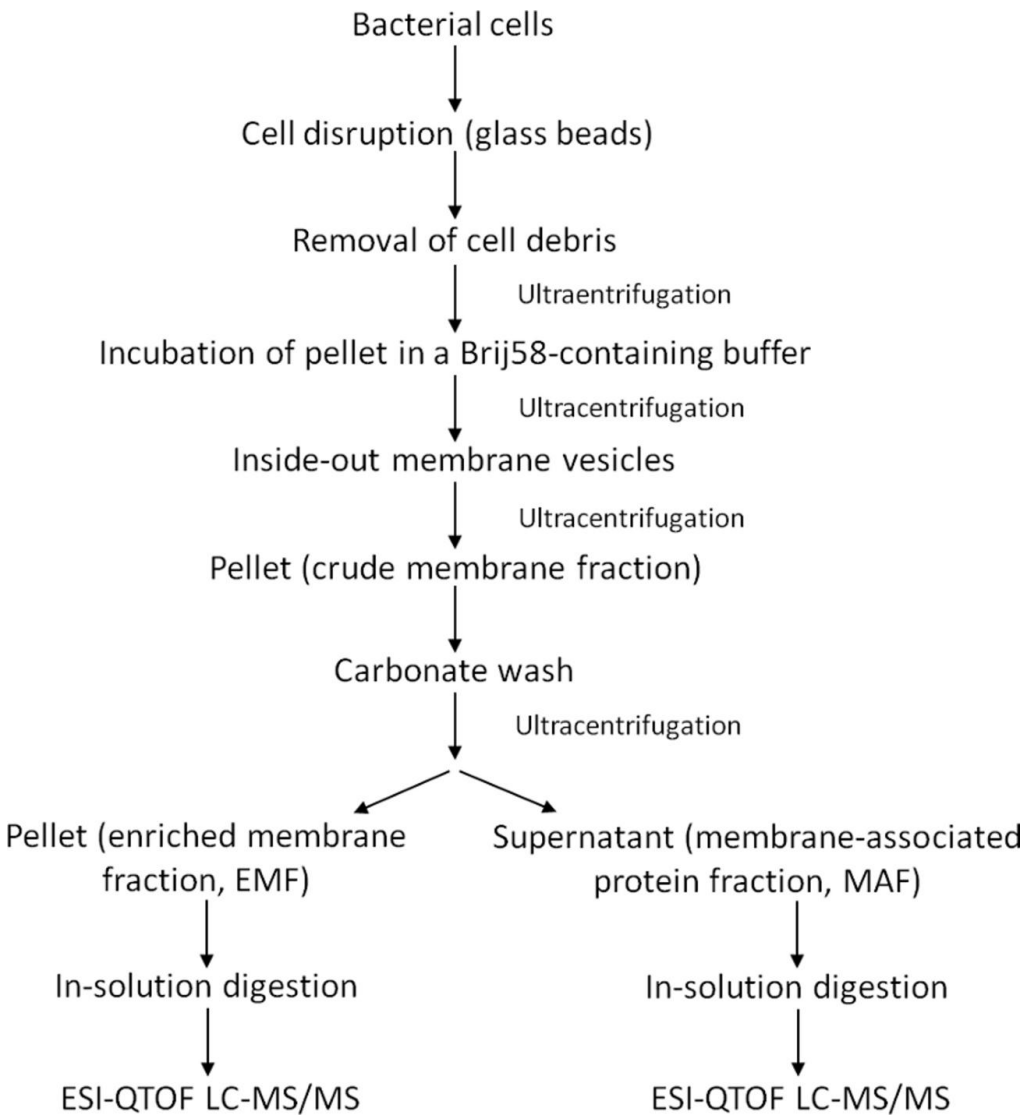


Figure 5.1. Schematic illustration of the protocol used to in membrane protein fractions from *B. animalis* subsp. *lactis* BB-12.

classification domains. Ninety-one, accounting for 36.7% of all identified proteins, were classified as MP, whereas cytoplasmic proteins (CP) and ribosomal proteins (RP) represented 31.5% and 18.1%, respectively (all RP were also classified as CP) and 36.7% of the proteins were not assigned to a cellular localization category.

Membrane samples containing CP and RP commonly appearin membrane proteomics (Klein *et al.* 2005, Li *et al.* 2011], and were previously reported to each account for 16.3% of the proteins identified from the membrane fraction of a bifidobacterial strain (Ruiz *et al.* 2009). RP

may be associated – directly or indirectly – to membrane proteinsor to the lipid bilayer (Rabilloud 2007), and have been shown to be involvedin translocation of MP and secreted proteins (Luirink and Sinning 2004), sensing of environmental changes in bacteria like *E. coli* (Van Bogelen and Neidhardt 1990) and to exhibit immunomodulatory properties associated with gonococcal invasion (Spence and Clark 2000). These findings concur With the identification of RP on bacterial cell envelope (Tjalsma *et al.* 2008). An alternative explanation for the presence of ribosomal proteinsin membrane samples which should be considered in the present study was suggested by Klein *et al.* (Klein *et al.* 2005), who documented a specific binding of ribosomal contaminants to the membranes, hypothesized to be caused by low ionic strength throughout membrane isolation facilitating electrostatic/hydrophobic association of RP with membranes.

The identification of CP (e.g. enolase, glyceraldehyde 3-phosphate dehydrogenase and elongation factor Tu) in the membrane fractions is in agreement with these proteins beingregarded as moonlighting proteins, i.e. CP displayed at the cellsurface or being secreted through a yet unidentified mechanism (Jeffery 2003) and which are reported to act as mucosa adhesion promoting factors in other bifidobacteria (reviewed in [Sánchez *et al.* 2010]).

In addition to the presence of CP and RP in the membrane samples, resolving membrane proteomes are not a trivialtask. Common challenges are related to solubilizing of highly hydrophobic proteins, especially while utilizing an in-solution digestion approach. The amphipathic nature of MP that protrude outside the membrane can also cause undesired precipitation in both
hydrophobic and hydrophilic solvents (Rabilloud 2007), and application of detergents that facilitate solubilization of hydrophobic proteins is in most cases incompatible with subsequent reverse-phased-based chromatographic fractionation like LC–MS (Rabilloud 2007) and ionic detergents like SDS that interfere with fractionation using IEF.

In the present study, two consecutive approaches aimed to enrich for membrane proteins were applied. These approaches conferred treatment of the crude membrane samples with the detergent Brij 58, with the purpose to obtain inside-out vesicles (Johansson *et al.* 1995) that increase the exposure of membrane-associated proteins to the carbonate buffer they are subsequently washed with. The latter interferes withthe attachment of these proteins to the membrane and thusreduces their occurrence in the resulting membrane samples (Molloy 2008). The present study originally conferred comparative analysis of the protein content of the EMF and the category names correspond to the number of category assignments and the percentage of assignments assigned to the different categories of the total number of proteins identified.

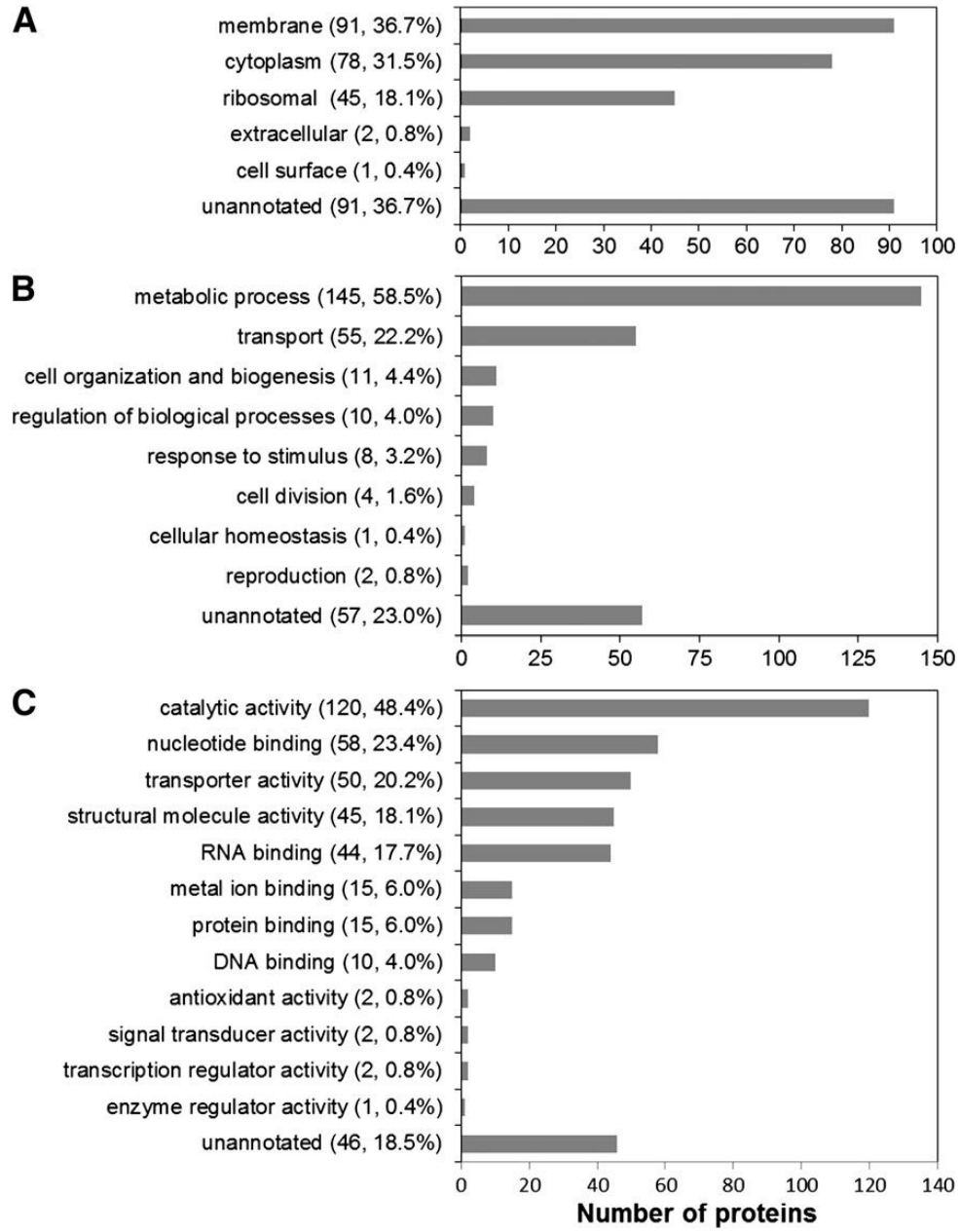


Figure 5.2. Gene Ontology classifications of the proteins identified from the membrane fractions of *B. animalis* subsp. *lactis* BB-12. A, Subcellular component; B, Biological processes; C, Molecular functions. Proteins were in some cases (82 of the proteins indentified) assigned to more than a single category. Numbers and percentage designated to the right of **Figure 5.2. Gene Ontology classifications of the proteins identified from the membrane fractions of *B. animalis* subsp. *lactis* BB-12. A, Subcellular component; B, Biological processes; C, Molecular functions. Proteins were in some cases (82 of the proteins indentified) assigned to more than a single category.** **Numbers and percentage designated to the right of the category names correspond to the number of category assignments and the percentage of assignments assigned to the different categories of the total number of proteins identified.**

MAF, which was expected to demonstrate enrichment for integral membrane proteins in the former compared with the latter. Unfortunately, inconsistent quality of the obtained tandem MS spectra made such a comparison infeasible. Comparing the number of TMSs-containing proteins as a part of the total proteins identified in the merged EMF and MAF to those of the total proteome – 36.3% and 25.1%, respectively,which are similar to the 36.2% and 26.2%, respectively,obtained in a previous analysis of membrane fractions of *B. longum* NCIMB 8809 (Ruiz *et al.* 2009) – shows that the applied experimental protocol gave rise to enrichment for membrane proteins. One can presume that this enrichment would have been even more distinct if the protein content in the EMF and MAF could have been analyzed separately, as observed in spectra of satisfying quality (Gilad *et al.*, unpublished data), implicating the efficacy of the protocol for membrane proteinisolation.

The biological processes GO classification (Fig. 5.2B) showed that the majority of the identified proteins are assigned to the categories – in decreasing order of occurrence – "metabolic processes", "transport" and "cell organization and biogenesis". Similar results were obtained in a study of the liposoluble proteome of *Mycoplasma agalactiae*, where nearly 40% of the proteins identified were classified under various metabolic processes and approx. 16% of the proteins identified were classified under the "transport" category (Cacciotto *et al.* 2010). Noticeably, nearly onefourth of the identified proteins are unannotated. The predominance of proteins associated with metabolic processes stems from assignment of RP to this category and from the presenceof CP in the membrane fractions, including nearly all the enzymes of the fructose-6-phosphate shunt, also known as the bifid shunt,which is the central pathway of carbohydrate catabolismin bifidobacteria. Proteins predicted to play a role in cell wall biosynthesis account for 5 of the 11 proteins in the "cell organization and biogenesis" category. In addition to the 55 proteins belonging to transport processes (hence MP), manual inspectionof identified remaining proteins not annotated to this category by the GO classification showed that when other components of transport systems (like ATP- and solute-binding proteins [SBP]) are included, the number of transport-related proteins is 67 (Section 5.3.2; Tables 5.1–5.2). When including alsothe 12 proteins involved in protein translocation (Table 5.3), thetotal amount of identified MP is 79, which is comparable to 78 transport-related proteins identified in the membrane fraction of *B. longum* NCIMB 8809 (Ruiz *et al.* 2009). Of the 79 identified BB-12 transport-related proteins (Tables 1–2), 37 were homologous (BLASTP sequence alignment E values $< 1\times10^{-10}$) to identified *B. longum* NCC2705 transport-related proteins (Supplemental Table A.4).

The GO classification Molecular functions (Fig. 5.2C) gave rise to a large number of proteins associated with the "catalytic activity" and "transport" categories. The high representation

in the categories "structural molecule activity" and "RNA binding "is associated with the large number of RP assigned tothese categories. The "nucleotide binding" category consists of ATP-binding proteins, chaperons such as DnaK, DnaJ andthe 60 kDa chaperonin GROEL, as well as proteins involved in transcription and translation, e.g. elongation factor Tu, DNA topoisomerase I, tyrosyl-tRNA synthetase and HelY (helicase). The distribution of the number of TMSs among the identified proteins is listed in Table 5.4. The tendency of the distribution of TMSs was found to be similar to that found for the whole membrane proteome of the bacterium, as exemplified by the relatively large number of proteins with 6 predicted TMSs (Table 5.4) that coincides with the large number of ABC permeases usually having 6 TMSs (Biemans-Oldehinkel *et al.* 2006). Of the identified TMSs-containing proteins, 34.2% are predicted to possess only 1–2 TMS(s) (Table 5.4), including SBP belonging to ABC transporter systems, and adhesion proteins typically having either a C- or an N-terminal anchor, or anchors at both termini.

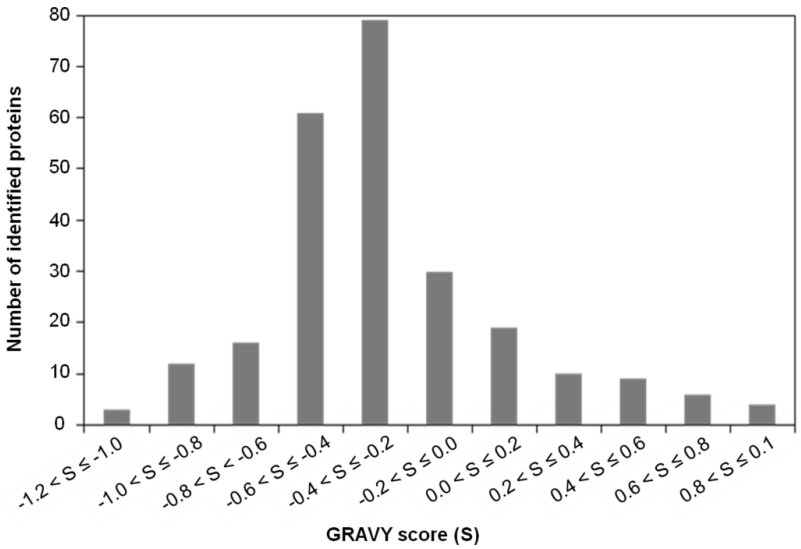


Figure 5.3. Distribution of the GRAVY scores (obtained at www.gravy-calculator.de/) of the transmembrane segments-containing proteins from *B. animalis* subsp. *lactis* BB-12 identified in the present study.

The GRAVY scores of the identified proteins (Fig. 5.3) indicate their hydrophobicity (Kyte and Doolittle 1982). More than two thirds of the identified proteins are not classified as MP, therefore the majority have negative GRAVY scores; the overall average value is −0.2. The most hydrophobic protein identified is the hypothetical membrane spanning protein (BIF_01840) predicted to have 6 TMSs, which attained a score of 0.99, whereas the proteinwith most TMSs (14) − MviN (virulence factor; BIF_01669), obtained a score of 0.13. This incompatibility between the

number of predicted TMSs and GRAVY scores of these two proteins is related to the fact that even though BIF_01669 has more than twice as many TMSs, the markedly smaller size of BIF_01840 (less than one sixth of the number of amino acids of BIF_01669), gives rise to a higher occurrence of TMSs per amino acid in BIF_01840, and hence a higher GRAVY score. A similar observation was reported by Santoni *et al.* (2000).

5.3.2 Transport proteins identified in BB-12

The predominant groups of 79 proteins identified in the membrane fractions and predicted to play a role in transport is described below according to their predicted substrates.

5.3.2.1. Oligosaccharide transport

Twelve proteins – all ABC transporters – are predicted to beinvolved in oligosaccharide uptake (Table 1), comprising fiveSBP, five permeases and two ATP-binding proteins. Along with the genes (BIF_00257 and BIF_00258) coding for permease proteins, the gene (BIF_00212) encoding a sugar-binding protein belongs to a putative cluster containing 9 genes encoding proteins related to XOS catabolism. Eight of these genes were up-regulated when BB-12 was grown with XOS compared with glucose as carbon source, and the abundance of three of the corresponding proteins, including the gene product ofBIF_00212, was differentially increased (Gilad *et al.* 2010).

The genes coding for the permeases MalC and MalG (BIF_1619 and BIF_01618, respectively) and the maltose/maltodextrin-binding protein (BIF_01620) are positioned in tandem on the chromosome of BB-12. These genes are partof a predicted gene cluster whose constituents are related to utilization of oligosaccharides. Noticeably, the transcription of the genes encoding for MalC and MalG increased 6-and 13-fold, respectively, in BB-12 grown on FOS compared with glucose (Gilad O, unpublished data). Their homologs from *B. longum* NCC2705 belong to putative oligosaccharide operons and also the gene coding for a sugar ABC transporter solute-binding protein (BL1164) homologous to BIF_01620, was up-regulated on FOS (Parche *et al.* 2007). Based on these observations, it was hypothesized that the corresponding proteins participate in oligosaccharide uptake. A fifth permease, MsmG (BIF_00747), is homologous to a *Streptococcus mutans* transporter involvedin uptake of melibiose, raffinose, and isomaltotriose (Russell *et al.* 1992). A *B. longum* NCC2705 gene encoding a protein homologous to MsmG was up-regulated on maltose (Parche *et al.* 2007), implying the potential oligosaccharide transport activity of this BB-12 permease.

Table 5.1. *B. animalis* subsp. *lactis* BB-12 proteins identified in the present study and predicted to be involved in oligosaccharide transport.

Locus tag	Acc. number	Description	*B. longum* homolog (BLASTP E value)	Additional information
BIF_01681[a]	ADC84994	Sugar transport ATP-binding protein	BL0673 (0), msiK, ATP binding protein of ABC transporter for sugars	Up-regulated on XOS compared to glucose in intracellular 2D-DIGE analysis (Gilad *et al.* 2010); Homologous ($7E^{130}$) to msiK from *Streptomyces coelicolor* A3(2), a universal ATPase of several ABC-type carbohydrate transporters (Russell *et al.* 1992)
BIF_01629[a]	ADC84915	ABC transporter ATP-binding protein	BL0197 ($2E^{-30}$), possible ATP binding protein of ABC transporter	
BLA_0461[b]	ACL28760	Probable solute binding protein of ABC transporter system for sugars	BL1164 (0), probable solute binding protein of ABC transporter system for sugars	*bl1164* was up-regulated on FOS and is predicted to belong to FOS/lactose operon (Parche *et al.* 2007)
BIF_00212[a]	ADC85538	Sugar-binding protein	BL1163 ($5E^{-16}$), probable solute binding protein of ABC transporter system for sugars	*bl1163* is located vicinal to *bl1164*, which was up-regulated on FOS (Parche *et al.* 2007)
BIF_00257	ADC85539	Transporter	BL0189 ($5E^{-37}$), sugar permease of ABC transporter system	*bif_00257* up-regulated on XOS (Gilad *et al.* 2010)
BIF_00258	ADC85540	Transporter	BL1523, msmG ($4E^{-34}$), sugar permease of ABC transporter system	*bif_00258* up-regulated on XOS (Gilad *et al.* 2010); *bl1523* up-regulated on raffinose (Parche *et al.* 2007) and GOS (Wang and Reeves 1994)
BIF_01620	ADC84948	Maltose/malto-dextrin-binding protein	BL1164 ($9E^{-11}$), probable solute binding protein of ABC transporter system for sugars	*b1164* up-regulated on FOS (Parche *et al.* 2007)
BIF_01619	ADC84947	MalC	BL0424 ($3E^{-20}$), sugar permease of ABC transporter system	*bl0424* belongs to a FOS operon, vicinal gene up-regulated on FOS (Parche *et al.* 2007)
BIF_01618	ADC84946	MalG	BL0190 ($2E^{-30}$), sugar permease of ABC transporter system	*bl0190* belongs to an ABC transporter-type operon with unknown function (Parche *et al.* 2007)
BIF_00747	ADC84953	MsmG	BL0144 ($1E^{-145}$), permease of ABC transporter possibly for oligosaccharides	Homologous ($5E^{-35}$) to msmG from *Streptococcus mutans*, which is involved in uptake of melibiose, raffinose, and isomaltotriose (Santoni *et al.* 2000); *bl0144* up-regulated on maltose (Parche *et al.* 2007)
BIF_00469	ADC84507	Maltose/malto-dextrin-binding protein	BL0141 ($1E^{-140}$), possible solute binding protein of ABC transporter	*bl0141* up-regulated on maltose (Parche *et al.* 2007)
BIF_01592	ADC84981	Raffinose-binding protein	BL1521, msmE (0), sugar binding protein of ABC transporter system	*bl1521* up-regulated on lactose (Parche *et al.* 2007)

Footnotes: [a] Proteins whose homologs were identified in the cell-envelope of *B. longum* NCIMB 8809 (Ruiz *et al.* 2009). [b] The glycerol-binding protein Bla_0461 belongs to *B. animalis* subsp. *lactis* AD011, a strain highly related to BB-12, but BLASTP search of the amino acid sequence of the protein does not give any result from the latter strain.

Finally, 15 ABC transporter ATP-binding proteins were identified (Tables 5.1–5.2). Two of these are the ABC transporter ATP-binding protein (BIF_01629) and sugar transport ATP-binding protein (BIF_01681). The latter is a homolog of the ABC transporter ATP-binding protein of *Streptomyces coelicolor* A3(2) that acts as ATPase subunit in several ABC sugar transporters (Bertram *et al.* 2004). Since BIF_01681 was over-produced on XOS, although induction at the transcriptomic level was not observed (Gilad *et al.* 2010), it is possible that this protein has a similar role in BB-12.

5.3.2.2. Miscellaneous transport proteins

Apart from proteins predicted to be involved in oligosaccharide uptake, another 63 transport proteins were identified (Table 5.2), which are associated with amino acid, inorganic ion (manganese, copper, potassium and calcium), nucleotide and phosphate transport. A few of the SBP identified in the present study, i.e. BIF_00212, BIF_00469, BIF_01620, BIF_01087, BIF_01234 and BIF_00737, were also identified in the extracellular proteome of BB-12 (Gilad *et al.* 2011). Accordingly, these proteins arepredicted to contain N-terminal membrane-anchors.A noteworthy finding is the detection of 7 of the 8 subunitsof the F_1F_0-ATP-synthetase complex (with the exception of the C chain subunit). This membrane-embedded enzyme complex, encoded by the ATP operon, which is highly conserved among bacteria (Ventura *et al.* 2004) catalyzes the ATP-driven translocation of protons to the exterior of the cell. The identification of this complex is in agreement with other studies where the acid inducibility of the ATP operon and the increased production and activity of the enzyme complex under acidic conditions in *B. animalis* subsp. *lactis* strains reflected a crucial role in responseand tolerance to low pH (Matsumoto*et al.* 2004). The ATP-synthetase complex was also documented to be involved in bile salt tolerance (Sánchez *et al.* 2006). The dependence, however, of the acid tolerance of BB-12 on the ATPase complex has yet to be corroborated by functional studiesof the ATP operon.

Another important industrial application-related property of BB-12 is the biosynthesis and secretion of polymeric capsule like structures composed of exopolysaccharides (EPS). These biopolymers possess texture enrichment and stabilizing properties desirable in a variety of fermented milk products. Some EPS demonstrated *in vitro* health-promoting effects like antitumor and immunomodulatory activities (Ruas-Madiedo *et al.* 2002). Studies that aimed at deciphering their role in bacterial adhesion to human intestinal mucus, showed, however, that EPS interfere with adhesion of probiotic bacteria and to enhance adhesion of pathogenic bacteria (Ruas-Madiedo al. 2006). In the present study, four proteins potentially involved in export and biosynthesis of EPS were identified, including a polysaccharide export permease protein (BIF_01639), an ATP binding

Table 5.2. Transport-related proteins identified in the membrane fractions of *B. animalis* subsp. *lactis* BB-12.

Locus tag	Acc. number	Description
Amino acid transport		
BIF_00040[a]	ADC84522	OppC (oligopeptide transport system permease protein)
BIF_00083[a]	ADC86102	ABC transporter ATP-binding protein
BIF_00164	ADC85464	Glutamate/gamma-aminobutyrate antiporter
BIF_00455[a]	ADC84651	Glutamine-binding protein
BIF_00580[a]	ADC85577	Amino acid ABC transporter permease protein
BIF_00737[a]	ADC84524	OppA (oligopeptide-binding protein)
BIF_00778[a]	ADC85696	Glutamate-binding protein
BIF_00779[a]	ADC85695	GluA (glutamate transport ATP-binding protein)
BIF_00905	ADC85575	ABC transporter amino acid-binding protein
BIF_01038[a]	ADC85578	Amino acid transport ATP-binding protein
BIF_01105[a]	ADC84669	OppA (oligopeptide-binding protein)
BIF_01811	ADC85576	ABC transporter amino acid-binding protein
BIF_01969	ADC85179	ABC transporter ATP-binding protein
BIF_02143[a]	ADC84521	OppD (oligopeptide transport ATP-binding protein)
Inorganic ion transport		
BIF_00250[a]	ADC84989	Calcium-transporting ATPase
BIF_00651[a]	ADC85772	CorA
BIF_00921	ADC85757	KtrA (potassium uptake protein)
BIF_01086	ADC86007	Manganese transport system ATP-binding protein
BIF_01087	ADC86005	Manganese-binding protein
BIF_01137[a]	ADC85118	Transporter
BIF_01143	ADC86059	Copper-exporting ATPase
BIF_01234	ADC85371	Manganese-binding protein
BIF_01626	ADC84918	Kup system potassium uptake protein
BIF_01656	ADC85070	CorC (magnesium and cobalt efflux protein)
BIF_01885	ADC84866	PacS (cation-transporting ATPase)
Nucleotide transport		
BIF_00363[a]	ADC85734	Hydroxymethylpyrimidine transport ATP-binding protein
BIF_01727[a]	ADC86064	Uracil permease
Phosphate transport		
BIF_00142[a]	ADC85369	PstB (phosphate transport ATP-binding protein)
BIF_01096	ADC85367	PstC (phosphate transport system permease protein)
BIF_01097[a]	ADC85366	Phosphate-binding protein
Exopolysaccharide secretion and biosynthesis		
BIF_01639	ADC84907	Polysaccharide export ATP-binding protein
BIF_01640	ADC84908	Polysaccharide export ABC transporter permease protein
BIF_01476	ADC84799	Chain length regulator (capsular polysaccharide biosynthesis)
BIF_00944	ADC84780	Undecaprenyl-phosphate galactosephosphotransferase

ATPase complex subunits		
BIF_00327[a]	ADC84817	ATP synthase B chain
BIF_00544	ADC84818	ATP synthase gamma chain
BIF_01337[a]	ADC84823	ATP synthase A chain
BIF_01338[a]	ADC84821	ATP synthase B chain
BIF_01339[a]	ADC84820	ATP synthase delta chain
BIF_01340[a]	ADC84819	ATP synthase alpha chain
BIF_01897	ADC84816	ATP synthase epsilon chain
Miscellaneous transport proteins		
BIF_00037[a]	ADC84681	Multidrug/protein/lipid ABC transporter family, ATP-binding and permease protein
BIF_00411[a]	ADC85528	Transporter
BIF_00625	ADC85619	Transporter
BIF_00659[a]	ADC85833	Aquaporin
BIF_00743[a]	ADC84628	Transporter
BIF_00823[a]	ADC85571	FtsE (cell division protein)
BIF_00824[a]	ADC85572	FtsX (cell division protein)
BIF_01025	ADC85485	Transporter
BIF_01533	ADC84682	Multidrug resistance ABC transporter ATP-binding and permease protein
BIF_01630[a]	ADC84914	ABC transporter permease protein
BIF_01747[a]	ADC86103	ABC transporter substrate-binding protein
BIF_01969	ADC85179	ABC transporter ATP-binding protein

Footnotes: [a] Proteins whose homologs were identified in the cell-envelope of B. longum NCIMB 8809 (Ruiz *et al.* 2009). [b] BIF_00327 is annotated as an ATP synthase B chain, yet it differs from the β chain protein of B. lactis DSM 10140, a strain very closely related to BB-12, in only two amino acids, such that this may be an imprecise annotation.

protein (BIF_01640), a chain length regulator (BIF_01476) and an undecaprenyl-phosphate galactosephospho-transferase (BIF_00944; EC 2.7.8.6) that is predicted to catalyze transfer ofgalactose from UDP-galactose to a polyprenyl carrier (Wang and Reeves 1994).

5.3.2.3. Proteins of the translocation machinery

In addition to transport proteins, another remarkable finding was the detection of 12 of the 20 proteins predicted to be associated with the protein translocation machinery (Table 5.3) that is conserved in numerous Gram-positive bacteria (MacConaill *et al.* 2003). In the present study, corresponding identified proteins include translocase subunits, chaperones, peptidyl-prolyl cis-trans isomerase (also identified in the extracellular proteome of BB-12 (Gilad *et al.* 2011) and a trigger factor. Using random translational fusions between potential translocation signals, and an export-specific reporter protein designed to capture genes coding for secreted proteins, MacConaill *et al.* (MacConaill *et al.* 2003) demonstrated the functionality of this translocation machinery in *B. breve*

UCC2003. Since homologous proteins were found for the majority of the proteins that embody this translocation system, we postulate that a similar mechanism is also active in BB-12.

Table 3. Proteins of the putative translocation machinery in *B. animalis* subsp. *lactis* BB-12. Proteins identified in the present study are designated in boldface.

			Homology to *B. breve* UCC2003 protein	
Locus tag	Acc. number	Description	Sequence identity (%)	E value
BIF_00806[a]	**ADC86126**	**SecA**	**81**	**0**
BIF_01461	ADC85333	SecE	77	$4E^{-38}$
BIF_01902	ADC85864	SecG	77	$1E^{-41}$
BIF_00901[a]	**ADC85423**	**Secy**	**85**	**0**
BIF_01141	ADC85321	Putative signal peptidase I	57	$4E^{-92}$
BIF_00335	ADC84614	Lipoprotein signal peptidase	59	$3E^{-61}$
BIF_01611	**ADC84939**	**DnaJ**	**66**	**$5E^{-137}$**
BIF_01614	**ADC84941**	**DnaK**	**89**	**0**
BIF_00120	**ADC86094**	**Trigger factor, ppiase**	**76**	**0**
BIF_00400	**ADC85681**	**60 kDa chaperonin GROEL**	**90**	**0**
BIF_00675	**ADC85383**	**10 kDa chaperonin GROES**	**85**	**$4E^{-60}$**
BIF_01612	ADC84940	GrpE (Heat shock molecular chaperone)	69	$2E^{-77}$
BIF_00707	**ADC85298**	**Signal recognition particle, subunit Ffh/Srp54**	**84**	**0**
BIF_01431	**ADC85211**	**FtsY**	**77**	**$3E^{-173}$**
BIF_01832[a]	**ADC85839**	**YajC (protein translocase subunit)**	**59**	**$2E^{-53}$**
BIF_01208[a]	**ADC85012**	**Inner membrane protein (OxaA/YidC)**	**68**	**$2E^{-173}$**
BIF_01746[b]	ADC86104	Peptidyl-prolyl cis-trans isomerase	77	$3E^{-98}$
BIF_00973	**ADC84814**	**Peptidyl-prolyl cis-trans isomerase**	**51**	**$4E^{-99}$**
BIF_01042	ADC84873	Sortase	62	$3E^{-144}$
BIF_00667	ADC84738	Sortase	39	$2E^{-67}$

Footnotes: [a] Proteins whose homologs were identified in the cell-envelope of *B. longum* NCIMB 8809 (Ruiz *et al.* 2009). [b]BIF_01746 was identified in extracellular proteome analysis of *B. animalis* subsp. *lactis* BB-12 (Sánchez *et al.* 2010).

5.3.2.4. Proteins associated with bacteria–host interactions

Eight of the 19 proteins secreted from cultures of BB-12 and postulated (based on sequence homology) to be involved in bacteria–host interactions (Gilad *et al.* 2011) were also identified in the present study (the corresponding proteins are designated in parenthesis below). These interactions consist of (among others) recruitment of human plasminogen (enolase and DnaK) that was demonstrated to play a role in binding of a highly-related bifidobacterial strain to human GIT (Gonzalez *et al.* 2008), attaching to human mucus and fibronectin (EF-Tu), adhesion to epithelial cells (a pair of manganese-binding proteins) or collagen (collagen adhesion protein), immunomodulatory effects (Transketolase) and fimbriae formation (collagen adhesion protein and hypothetical protein BIF_0999),which was shown to facilitate murinegut colonization in vivo [43].

The identification of these proteins,which are predicted to take part in adhesion to host tissues or toexert immunomodulatory response in host cells, is in agreement with their presence in the cell envelope recorded in the previous study (Gilad *et al.* 2011).

Table 4. Distribution of the number of transmembrane segments (TMSs) predicted (obtained by TMHMM2 at http://www.cbs.dtu.dk/services/TMHMM/) in the identified proteins and the predicted membrane proteome of *B. animalis* subsp. *lactis* BB-12.

	Number of proteins		
Number of TMSs	Present study	Total membrane proteome	Percent of protein identified
1	34	119	28.6
2	14	49	28.6
3	1	26	3.8
4	4	38	10.5
5	4	33	12.1
6	15	45	33.3
7	2	14	14.3
8	1	15	6.7
9	3	12	25.0
10	4	16	25.0
11	1	7	14.3
12	3	23	13.0
13	3	6	50.0
14	1	8	12.5
15	0	0	-
16	0	1	0.0
Total	90	412	21.8

5.4. Conclusions

The present study aimed at resolving the membrane proteome of the important probiotic strain *B. animalis* subsp. *lactis* BB-12, with emphasis on proteins involved in oligosaccharide uptake. A considerable number of MP were identified by LC-ESI-Q-TOF MS, including 41 proteins potentially involved inthe transport of oligosaccharides, amino acids, inorganicions, phosphate and nucleotides. In addition, 23 identified MP were predicted to participate in pivotal cellular processes like protein translocation, acid tolerance and EPS biosynthesis, as well as 13 MP associated with miscellaneous transport systems and 8 moonlighting and cell-envelope proteins predicted to play a role in bacteria-host interactions. The identification of these proteins may – supported by functional

studies – contribute to the comprehension of the molecular mechanisms underlying some of the central physiological traits associated with probiotic bacteria, i.e. utilization of a variety of nutrients (e.g. oligo-saccharides, amino acids, inorganic ions, nucleotides and phosphate) available in the host GIT through a battery of transport systems with diverse substrate specificity, maintaining viable cellular pH by an ATP-driven proton pump and reducing adhesion of probiotic strains to host cells via secretion of EPS. Better insight into mechanisms can assist in designing probiotic strains with desirable physiological characteristics, i.e. improved acid and bile tolerance and lower production of EPS, as well as enhanced health-promoting properties, which can serve as a platform for the design of a so-called "super" probiotic strain.

5.5. Materials and methods

5.5.1. Experimental design

Cultures of *B. animalis* subsp. *lactis* BB-12 (3×1 L) were grown in a de Man, Rogosa and Sharpe (MRS)-reconstituted medium supplemented with 2% of either glucose or XOS as described (Gilad *et al.* 2010) and harvested at late-exponential phase and an OD of 1.1 (8 h growth with XOS and 9 h with glucose). Enriched membrane fractions (EMF) and membrane-associated fractions (MAF) were isolated (see Section 5.5.2 and Fig. 5.1) and analyzedby LC–MS/MS. Tandem MS peak lists files obtained forthe different samples were merged into a single peak list which was used for protein identification using Mascot Daemon (Matrix Science Ltd., London).

5.5.2. Isolation of membrane fractions

Cells were harvested (6600×g, 20 min; all centrifugations werecarried out at 4 °C), washed twice in 25 mM potassium phosphate pH 7.0 and kept at −80 °C. Pellets were resuspended in 5 mL homogenization buffer (0.2 M Tris–HCl pH 7.5, 4.2 mM EDTA, 42% glycerol), added with glass beads (425–600 μm, Sigma-Aldrich) and subjected to cell disruption (5 cycles of 1 min whirling at 12,000 rpm and 1 min cooling on ice between cycles). Subsequently, homogenization buffer (15 mL) was added to increase the sample volume and thereby reducing loss of membrane vesicles in the centrifugation (8000×g, 15 min), followed by ultracentrifugation (100,000×g, 60 min; Ti60 rotor; Beckman-Coulter, Fullerton, CA) of the supernatant. To improve efficacy of the subsequent carbonate wash, inside-out membrane vesicles were prepared by resuspending the pellet containing crude membrane fraction in 1 mL "inside-out buffer" (150 mM KCl, 25 mM sodium acetate pH 4.0, 0.01% Brij 58 [Sigma-Aldrich, Saint Louis, MI] [11]), kept onice for 30 min, and ultracentrifuged (100,000×*g*, 30min). The resulting pellet containing "inside-out vesicles" was resuspended in 2 mL freshly made 0.1 M Na_2CO_3 using a manual homogenisator, transferred to 2

mL tubes, kept on ice (30 min) and centrifuged (20,000×g, 30min). A volume of 1.5 mL resuspension buffer (20% glycerol, 0.1 M Tris–HCl pH 7.5, 1 mM EDTA) was added to the pellet containing integral MP and membrane-associated proteins not removed by the two washing steps (designated as "Enriched Membrane Fraction "[EMF]) and, along with the supernatant that contained membrane-associated proteins (designated as "Membrane-Associated Fraction" [MAF]), the samples were subjected to protein determination (Bradford 1976). The isolation of EMF and MAF is schematically depicted in Fig. 5.1.

5.5.3. Protease digestion

EMF andMAF (20 µg) isolated fromthe XOS and glucose cultureswere precipitated in chloroform/methanol (Wessel and Flügge 1984), resuspended in 40 µL 6 M urea, 2 M thiourea, 20 mM DTT and reduced and alkylatedas described (Thingholm *et al.* 2008). Forty-five microliter 100 mM NH_4HCO_3 were added to the samples prior to incubation with 0.4 µg lysylendopeptidase (Wako pure Chemical Industries LTD, Osaka, Japan) at room temperature (RT) for 3 h, followed by ×5 dilution with 50 mM NH_4HCO_3, addition of 0.4 µg of modified trypsin (Promega) and incubation at RT for 16 h. Concentration and desalting of peptides was performed as described for R3 Columns (Thingholm *et al.* 2008) using POROS Oligo R3 Reversed-Phase resin dissolved in acetonitrile (ACN) and packed on topof a C18 membrane disk into a P10 pipette tip. Columns were washed with ACN and equilibrated with 0.1% [v/v] TFA (all the percent values given in Sections 2.3–2.4 are in [v/v]). Digests were acidified with formic acid (FA), loaded on to the columns, washed with 0.1% TFA and eluted in two steps by 30 µL 50% ACN/0.5% TFA followed by 30 µL 70% ACN/0.5% TFA. The two eluates were pooled, lyophilized, resuspended in 1 µL 100% FA and diluted with 19 µL water prior to LC–MS.

5.5.4. Liquid chromatography coupled with tandem massspectrometry

Peptide separation was achieved using an LC-Packings Ultimate3000 nanoflow system (LC Packings, Amsterdam). Peptides were loaded at a flow rate of 5 µL/min onto a custom-made 1 cm precolumn (100 µm inner diameter) of fused silica with kasil-frits retaining the Reprosil C18, 3.5 µm reversed-phase particles (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). Nanoflow RP-HPLC was then performed at a flow of 0.25 µL/min through a custom-made 12 cm analytical column (75 µm in nerdiameter), packed with Reprosil C18, 3.5 µm reversed-phase particles. Peptides were eluted directly into the ESI source of a Q-TOF Premier tandem mass spectrometer (Waters/Micromass,Manchester, UK) using a 0–35% gradient of solvent A (0.1% FA) and solvent

B (95% ACN, 0.1% FA) applied for either 30, 60, or 120 min. Mass- and charge-dependent collision energies were used for peptide fragmentation.

5.5.5. Data analysis

Peak list files were created using VEMS v 3.0 (Matthiesen *et al.* 2005) and merged into a single peak list that was searched against the genome sequence of BB-12 (GenBank accession no. CP001853, 1612 ORFs, 587,905 amino acid residues) (Garrigues *et al.* 2010) as taxonomy entry, using anin-house licensed Mascot server version 2.2 (29th September 2010). The search parameters applied allowed a single missed cleavage, cysteine carbamidomethylation as fixed modification and methionine oxidation as variable modification. Peptide and fragment ion mass tolerance were set at ±20 ppm and ±0.2 Da, respectively. No restrictions with respect to protein mass andpI were made. A protein hit was taken into consideration when two unique peptides with statistically significant scores (P<0.05) were obtained. The false discovery rate for peptides above identity threshold was calculated using the decoy feature of Mascot and resulted in a value of 1.68%. For cases with only two peptides, the identification was validated manually basedon visual inspection of the tandem spectra.

5.5.6. Bioinformatic analysis

The proteomics data mining andmanagement software ProteinCenter (Proxeon Bioinformatics, www.proxeon.com) was used to sort the identified proteins with respect to their predicted Cellular components, Molecular functions and Biological processes according to The Gene Ontology (GO; (Ashburner *et al.* 2000) classification system. Protein Center also supported prediction of the presence of signal peptide and transmembrane segments (TMSs). GRAVY scores are calculated according to the sum of hydropathy values of all amino acids divided by the protein length were obtained at www.gravy-calculator.de/. Protein sequence alignments were performed using BLASTP 2.2.25 with the All non-redundant Gen-Bank CDS translations+PDB+SwissProt+PIR+ PRF (excluding environmental samples from WGS projects) database. The BLASTP scores of the proteins predicted to be involved in the translocation machinery (Table 5.3) represent alignments with proteins from *Bifidobacterium breve* UCC2003. Since the genome sequence of *B. longum* NCIMB 8809 is not publicly accessible, *B. longum* NCC2705 was used for comparison with BB-12 (Supplemental Table 2). GenBank accession numbers of the proteins are designated in Tables 5.1−5.3 and Supplemental Tables A.4 –A5.

Concluding remarks and perspectives

Due to their various health-benefiting effects, probiotic bacteria in general and bifidobacteria in particular have been attracting growing interest from both the scientific and industrial communities. In spite of extensive studies of a multitude of aspects concerning the physiology of these bacteria and the mode of action underlying probiotic effects, these mechanisms are still far from deciphered.

The present PhD study had two primary, and to some extent, interconnected objectives. The first aimed at broadening the knowledge regarding the proteins involved in interactions between the probiotic strain *Bifidobacterium animalis* subsp. *lactis* BB-12, and prebiotic carbohydrates that specifically stimulate the growth of probiotic bacteria in the human GIT. The second objective was to utilise proteome analysis in order to identify the proteins exported by the bacterium and those embedded in its membrane, respectively, and thereby shed more light into the proteins that come in contact with the environment surrounding the bacterium and are thus predicted to be associated with probiotic effects, as well as membrane proteins that take part in physiological processes essential for the survival of the bacterium in the GIT.

The present study began with screening of a wide battery of more than 40 carbohydrates for their ability to support the growth of BB-12. The results showed that BB-12 can ferment oligosaccharides like gluco- galacto- and to a lesser extent xylo- and fructo-oligosaccharides. Another noticeable finding was the higher growth rate obtained on all these substrates compared to glucose, which may be related to the utilisation of more efficient uptake of these oligosaccharides compared to glucose.

In the second phase of the study, cultures of BB-12 cultivated on XOS (an emerging prebiotic candidate chosen for further analysis) and glucose were subjected to comparative transcriptome and proteome analysis, supplemented with liquid chromatography- based analysis of XOS consumption. Based on the results, that showed up-regulation of a putative XOS-active gene cluster during growth on XOS, and on sequence homology searches, a model for XOS catabolism in BB-12 was established.

The third step in the study conferred extracellular proteome analysis, where the protein content of culture supernatants of BB-12 was mapped. This analysis gave rise to the identification of numerous proteins that were documented to be involved in host-interactions, as well as proteins associated with essential cellular processes as nutrient uptake and cell wall biogenesis and modification.

Finally, the membrane proteome of BB-12 was resolved using a gel-free approach that employed in-solution digestion accompanied with LC-MS/MS. Coinciding with the results of the

extracellular proteome analysis, proteins predicted to play a role in crucial physiological mechanisms such as acid tolerance, protein translocation, carbohydrate, amino acid an inorganic ions uptake and EPC biosynthesis were detected in the membrane fractions.

Regarding potential future perspectives of the present study, apart from the above-mentioned industrially-related elements, many aspects of various important biological processes in BB-12 are yet to be investigated. Such investigations can for instance confer studying some of the transport systems or the translocation machinery identified in the present study by recombinant production in a bifidobacterial plasmid or in *Lactococcus lactis*, coupled with activity assays. Similar methodologies may be applied for studies concerning the verification of the suggested model for XOS utilisation in BB-12, as well as investigation of the putative gene cluster by primer walking and Northern blotting.

With respect to the industrially-applicable significance of the present industrial-PhD project, the importance of the findings obtained in the present study can be roughly related to different components. Firstly, the knowledge regarding the carbohydrate fermentative capabilities of BB-12 can (and is practically already being used) for selecting potential substrates to be used either as prebiotic supplements in combination with BB-12 (a synbiotic preparation), or as growth stimulants at the production phase. Secondly, the considerable number of proteins identified in the extracellular and membrane proteome analyses can be used as promising targets for further investigation of the involvement of these proteins in probiotic effects or in some of physiological traits exhibited by BB-12 (e.g. acid tolerance and EPS production). This can also include mapping the proteins and enzymes involved in cell wall metabolism and their catalytic activities, as this process is of outmost importance to cell division, antibiotic resistance, immunomodulation, adhesion and also to texture-enrichment of dairy products by EPS. Elucidation of these mechanisms may have a substantial impact, both with respect to providing documentation for the molecular basis behind these effects, and with regard to the development of novel probiotic strains with enhanced physiological properties or the improvement of existing strains by genetic engineering.

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Appendix

- Publications included in the dissertation (See attached CD-ROM):

- Gilad O., Jacobsen S., Stuer-Lauridsen B., Garrigues C., Pedersen M. B., and Svensson B., Combined transcriptome and proteome analysis of *Bifidobacterium animalis* subsp. *lactis* BB-12 grown on xylo-oligosaccharides and a model of their utilization. *Applied and Environmental Microbiology* 76:7285-7291.

- Gilad O., Jacobsen S., Holm Viborg A, Stuer-Lauridsen B., and Svensson B. Extracellular proteome analysis of *Bifidobacterium animalis* subsp. *lactis* BB-12 reveals proteins with putative roles in probiotic effects. *Proteomics* 11:2503-2514.

- Gilad O., Hjernø K., Østerlund E.C., Margolles A., Svensson B., Stuer-Lauridsen B., Møller A.L.B. and Jacobsen S., Insights into physiological traits of *Bifidobacterium animalis* subsp. *lactis* BB-12 through membrane proteome analysis. *J. Proteomics* 75:1190-1200.

- Table A.1. Up-regulated genes (fold change FOS/glucose > 2) and down-regulated genes (fold change glucose/ FOS > 2) according to DNA microarrays analysis of samples derived from BB-12. See attached CD-ROM.
BL-Gene designates homologous proteins from *B. longum* NCC2705. #N/A: not annotated.

- Table A.4 (Supplemental Table 1 in the 3rd publication): **Protein identified in membrane fractions of *B. animalis* subsp. *lactis* BB-12 – see attached CD-ROM.**
Key for Gene Ontology (GO) classifications: M, membrane; C, cytoplasm; E, extracellular; CS, cell surface; s, signal sequence; T, transport; MP, metabolic process; CD, cell division; R, regulation of biological process; Rep, reproduction; OB, cell organization and biogenesis; RS response to stimulus; H, cellular homeostasis; CA, catalytic activity; MI, metal ion binding; RB, RNA binding; ST, signal transducer activity; TA, transporter activity; AA, antioxidant activity; DB, DNA binding; ER, enzyme regulator activity; PB, protein binding; NB, nucleotide binding; SM, structural molecule activity; TR, transcription regulator activity.

- Table A.5 (Supplemental Table 2 in the 3rd publication): **Comparison of the membrane proteomes of *B. animalis* subsp. *lactis* BB-12 and *B. longum* NCC2705 – see attached CD-ROM**.
Footnotes: Proteins designated in boldface were identified in the present study (BB-12) or in the study described in Ruiz *et al.* (2009; *B. longum*). The designated numbers of TMS(s) were determined using TMHMM2 according to the BB-12 proteins, unless no BB-12 homolog was found for the *B. longum* proteins.

Table A.2. **Proteins from BB-12 identified by MALDI-TOF MS and tandem MS from the differentially-abundant protein spots shown in Fig. 3.4.**

Spot No.	Fold change	ID	Protein description	Mascot PMF score[b]	E value	Sequence coverage (%)	No. peptides searched (matched)	Theor. (Obs.[c]) MW (kDa)	Theor. (Obs.) *pI*	Peptides identified by tandem MS[d]	Mascot tandem MS Ion score	E value
1	-1.8[a]	57	Aconitate hydratase	80	1.7×10^{-05}	18	94 (19)	98.3 (110)	4.8 (4.8)	K.AVIVESFER.I	23	2.9×10^{-4}
2	-1.6	155	Formate acetyltransferase	50	1.6×10^{-2}	11	23 (7)	92.0 (110)	5.1 (4.9)			
3	-3.0	332	Negative regulator of genetic competence clpC/mecB	141	1.3×10^{-11}	18	27 (15)	95.6 (100)	5.3 (5.5)			
4	1.8	1199	Acetyl-/propionyl-coenzyme A carboxylase α chain	109	2.0×10^{-8}	28	53 (16)	68.3 (75)	5.3 (5.6)	K.HSYLVTDAQDIPR.V	19	1.7×10^{-2}
		863	Acetolactate synthase large Subunit	63	9.1×10^{-4}	17	53 (10)	70.8 (75)	5.3 (5.3)			
5	1.8	405	β-xylosidase	117	3.2×10^{-9}	26	74 (17)	76.3 (70)	5.6 (4.4)	R.IDLDAADAAGVDPESIEAAR.T	44	5.0×10^{-5}
6	2.3	400	60 kDa chaperonin GROEL	263	8.1×10^{-24}	62	96 (32)	56.5 (70)	4.7 (4.7)	K.IIEYDEEAR.Q	33	9.3×10^{-4}
										R.FGLDLDFTEGMR.F	30	4.6×10^{-5}
										K.RTDDVAGDGTTTATVLAQSLVHEGLK.N	60	6.2×10^{-8}
										K.IAEALDKVGQDGVVTVEDNNR.F	74	2.7×10^{-8}
7	2.0	400	60 kDa chaperonin GROEL	167	3.2×10^{-14}	39	54 (17)	56.5 (70)	4.7 (4.7)			
		1321	PhosphOGlucomutase	121	1.3×10^{-9}	27	54 (15)	60.6 (70)	4.9 (4.7)			
8	1.8	177	ftsZ	87	3.0×10^{-6}	41	89 (13)	43.5 (60)	4.4 (4.2)	R.LLELDDSSIGIVDAFR.T	37	3.7×10^{-4}
										K.LAQANEDAQAAASAPQR.T	18	3.4×10^{-2}
										K.LAEVNQAVGFVR.E	18	2.7×10^{-2}
		1629	ABC transporter ATP-binding Protein	29	2.0×10^{0}	18	88 (7)	38.6 (60)	4.3 (4.2)	K.QFDNIIDLR.- R.FGENTKADESTLADELDPVDAAR.L	23 25	1.2×10^{-2} 4.2×10^{-3}
9	2.5	1979	Galactokinase	186	4.0×10^{-9}	43	66 (16)	44.9 (60)	4.5 (4.3)	R.APHQLNDGQYAQR.R	48	2.7×10^{-5}
										R.EVADLVNAQADPAAALDGVLDR.L	25	4.0×10^{-5}
										R.ILGVANLR.E	19	1.1×10^{-2}
										R.TYIALSPR.D	18	2.7×10^{-2}
										R.IAQAICDEFER.R	27	2.8×10^{-5}
10	13	501	Xylose isomerase	274	6.4×10^{-25}	53	95 (33)	52.8 (60)	4.7 (4.7)	K.AKEPTTHQYDFDAATACNFLR.A	107	2.9×10^{-11}
										K.LNLEGNHANLAGHTYQHEIR.V	117	3.0×10^{-12}

										K.LGAEYFCFHDRDIAPEGDTLR.E	29	1.2×10^{-3}
										K.EPTTHQYDFDAATACNFLR.A	104	4.4×10^{-11}
										R.TSFSAEDLFR.A	73	1.0×10^{-7}
11	13	501	Xylose isomerase	309	2.0×10^{-28}	51	100 (32)	52.8 (60)	4.7 (4.7)	K.LNLEGNHANLAGHTYQHEIR.V	123	8.6×10^{-13}
										R.LGAENYVFWGGR.E	46	3.3×10^{-5}
										K.LGAEYFCFHDR.D	28	2.7×10^{-2}
										K.AKEPTTHQYDFDAATACNFLR.A	129	1.8×10^{-13}
										R.RTSFSAEDLFR.A	55	6.2×10^{-6}
12	7.0	501	Xylose isomerase	261	1.3×10^{-23}	56	99 (34)	52.8 (60)	4.7 (4.7)	K.LNLEGNHANLAGHTYQHEIR.V	109	2.0×10^{-11}
										K.AKEPTTHQYDFDAATACNFLR.A	48	2.2×10^{-5}
										K.EPTTHQYDFDAATACNFLR.A	23	5.2×10^{-3}
13	2.2	137	Bacterial peptide chain release factor 2 (RF-2)	190	1.6×10^{-16}	50	77 (20)	42.0 (60)	4.7 (4.8)	R.ISPFDNQGR.R	37	3.1×10^{-4}
										M.AEFDYSQAISDAR.A	69	2.3×10^{-7}
										R.TGCETSQTQAVFDGDIDEFIQAGIR.W	71	1.1×10^{-7}
14	2.2	530	Phosphoglucosamine mutase	156	4.0×10^{-13}	50	94 (20)	48.2 (60)	4.9 (4.9)	R.VLLRPSGTEPLVR.V	19	8.7×10^{-3}
										R.DLTAQLALDLGDAAVR.V	73	7.1×10^{-8}
										R.YVLEEMLR.G[e]	22	1.6×10^{-2}
										R.ASGADLGVAFDGDADR.C	26	4.9×10^{-3}
		681	Argininosuccinate synthase	83	8.5×10^{-6}	38	94 (15)	45.9 (60)	4.8 (4.9)			
15	-8.8	1197	Enolase	60	1.6×10^{-3}	27	67 (8)	46.5 (50)	4.7 (4.2)			
16	-3.7	277	Phosphoglycerate kinase	47	3.0×10^{-2}	32	81 (9)	42.1 (50)	4.8 (4.3)	K.VVPELSLAPVAAR.L	19	1.2×10^{-2}
										K.DGDVVLLENVR.F	32	1.4×10^{-3}
		1197	Enolase	47	3.3×10^{-2}	25	81 (10)	46.5 (50)	4.7 (4.3)	R.LQFVGDDLFVTNPVR.L	40	1.8×10^{-4}
										R.GNPTVEVVLDTDDGAR.G	46	5.0×10^{-3}
		1636	Choline binding protein A	46	4.0×10^{-2}	39	81 (7)	19.7 (50)	9.3 (4.3)	M.AAIESVYAR.Q	18	3.8×10^{-2}
17	4.1	287	Transaldolase	115	5.1×10^{-9}	28	68 (13)	39.9 (40)	4.7 (4.5)			
		212	Sugar-binding protein	73	8.9×10^{-5}	24	68 (12)	48.4 (40)	4.8 (4.5)			
18	5.1	212	Sugar-binding protein	75	5.6×10^{-5}	29	52 (10)	48.4 (40)	4.8 (4.6)	K.AFSTIPASEPAR.A	38	2.8×10^{-4}
19	4.1	287	Transaldolase	169	2.0×10^{-8}	51	99 (21)	39.9 (40)	4.7 (4.6)	K.SWDSVLTDVQSGIDR.V	31	1.6×10^{-3}
		212	Sugar-binding protein	116	4.0×10^{-5}	42	99 (18)	48.4 (40)	4.8 (4.6)	K.AFSTIPASEPAR.A	50	2.1×10^{-5}

20	2.0	665	Hypothetical protein	67	3.2×10^{-4}	18	35 (9)	48.0 (40)	4.8 (4.8)	K.TSADFVQIAHALDR.A	33	7.9×10^{-4}
										K.SMTPAEELLIR.S[e]	27	4.4×10^{-3}
										R.DFGIPIVNKR.I	38	3.0×10^{-4}
21	-1.6	207	Choloylglycine hydrolase	140	1.6×10^{-11}	61	59 (14)	37.2 (35)	4.7 (4.7)	R.AAYNNTHYPTQSGENANVNR.L	88	1.5×10^{-9}
										R.FDDGQNNMYFGR.N	45	2.7×10^{-5}
										K.NVTIVGKPINDR.F	15	7.6×10^{-4}
22	-1.8	207	Choloylglycine hydrolase	100	1.6×10^{-7}	49	60 (12)	37.2 (35)	4.7 (4.7)	R.FDDGQNNMYFGR.N	45	5.1×10^{-5}
										K.NVTIVGKPINDR.F	15	3.8×10^{-2}
										R.NLDWSEDYGEK.I	49	2.2×10^{-5}
23	-2.3	207	Choloylglycine hydrolase	99	2.1×10^{-7}	41	96 (12)	37.2 (35)	4.7 (4.8)	R.FDDGQNNMYFGR.N	56	4.3×10^{-6}
										K.NVTIVGKPINDR.F	42	9.2×10^{-5}
24	-2.3	1306	Oxidoreductase	117	3.2×10^{-9}	30	37 (12)	37.5 (30)	5.4 (5.7)	R.YNIFDR.T	15	4.3×10^{-2}
25	-1.8	1306	Oxidoreductase	196	4.0×10^{-17}	46	45 (16)	37.5 (30)	5.4 (5.9)	R.IGHDDRFLNK.S	25	6.3×10^{-3}
										K.YLLSSLDQSLER.L	26	5.0×10^{-3}
26	-1.6	2081	(S,S)-Butane-2,3-diol dehy-Drogenase	112	1.0×10^{-5}	35	66 (12)	29.3 (20)	5.0 (5.3)	R.CETPDDVAGVVSFLVSDNAR.Y	71	8.3×10^{-8}
										K.DGFDVAVADLEGQR.Q	81	2.1×10^{-8}
										R.VAIVTGAAR.G	30	1.6×10^{-3}
										R.IEQEGRR.A	20	4.2×10^{-2}
										K.DGFDVAVADLEGQRQEAEETIHR.I	53	6.5×10^{-6}
										R.YVTGQTVIVDGGMQYR.-[e]	23	1.0×10^{-2}
27	1.6	1681	Sugar transport ATP-binding Protein	77	3.3×10^{-5}	22	51 (9)	41.4 (45)	5.7 (5.8)	M.AEVIFDHVTR.I	20	2.7×10^{-2}
										K.TTDTLMSDQNQLTTIR.V	16	3.9×10^{-2}
										K.AAEILDLTEFLDRKPK.A	15	1.6×10^{-2}
28	2.6	287	Transaldolase	162	1.0×10^{-13}	57	92 (20)	39.9 (40)	4.7 (4.6)	K.SWDSVLTDVQSGIDRVNG.-	61	1.3×10-6
										K.FAEDPRWAALEAK.G	17	4.6×10-2
		212	Sugar-binding protein	130	1.6×10^{-10}	45	92 (12)	48.4 (40)	4.8 (4.5)	K.AFSTIPASEPAR.A	61	1.5×10-6
										R.QYWENLAK.S	27	4.4×10-3

[a] Positive and negative values represent over- and under-expression in the XOS samples, respectively (with glucose as a reference).[b] Peptide mass fingerprinting (PMF) score obtained using the protein sequence database program Mascot (http://www.matrixscience.com) with the genome sequence of BB-12 as taxonomy entry. Scores > 45 are significant.
[c] Approximate values. [d] R. and K. denote cleavage at the C-terminal of an arginine and lysine residues, respectively. [e] Peptides oxidised at a methionine residue.

Table A.3. Proteins of BB-12 identified by MALDI TOF MS from the protein spots on the gel whose image is shown in Figure 4.1.

Spot #	Locus	GB acc. #	Protein ID	Mascot PMF Score[a]	E value	Sequence coverage (%)	# of matched peptides	# of searched peptides
1	BIF_00999	ADC84870	Hypothetical protein	72 (32)	$1.0e^{-04}$	23	9	55
1	BIF_00998	ADC84872	Collagen adhesion protein	46 (32)	$4.4e^{-02}$	11[c]	8	55
2	BIF_01178	ADC85287	Fibronectin-binding protein	148 (43)	$2.6e^{-12}$	14	30	54
3	BIF_00998	ADC84872	Collagen adhesion protein	117 (36)	$3.2e^{-09}$	23	15	42
3	BIF_01318	ADC84867	ClpB	67 (36)	$3.1e^{-04}$	17	11	42
4	BIF_00045	ADC85644	Protein Translation Elongation Factor G (EF-G)	144 (38)	$6.4e^{-12}$	35	20	61
4	BIF_00998	ADC84872	Collagen adhesion protein	69 (38)	$2.2e^{-04}$	18	12	61
5	BIF_00998	ADC84872	Collagen adhesion protein	99 (32)	$1.9e^{-11}$	13	9	17
6	BIF_00998	ADC84872	Collagen adhesion protein	92 (32)	$8.1e^{-10}$	12	8	15
7	BIF_00998	ADC84872	Collagen adhesion protein	76 (31)	$4.3e^{-05}$	16	11	34
7	BIF_01614	ADC84941	Chaperone protein	100 (31)	$1.8e^{-07}$	32	11	34
8	BIF_00963	ADC85857	Transketolase	67 (43)	$3.4e^{-04}$	16	9	50
9	BIF_00120	ADC86094	Trigger factor, ppiase	125 (39)	$5.1e^{-10}$	42	15	58
9	BIF_00400	ADC85681	60 kDa chaperonin GROEL	51 (39)	$1.2e^{-08}$	20	9	58
9	BIF_00315	ADC84590	Division specific D,D-transpeptidase	73 (39)	$8.1e^{-05}$	24	12	58
10	BIF_00592	ADC84840	Oxalyl-CoA decarboxylase	164 (38)	$6.4e^{-14}$	32	14	28
11	BIF_01677	ADC84998	Rpf protein precursor	152 (38)	$1.0e^{-12}$	26	14	30
12	BIF_00315	ADC84590	Division specific D,D-transpeptidase	196 (35)	$4.0e^{-17}$	46	20	49
13	BIF_00784	ADC85219	Hypothetical cytosolic protein	51 (44)	$1.3e^{-02}$	40	7	73
14	BIF_00825	ADC85573	Immunogenic secreted protein	59 (45)	$1.9e^{-03}$	14[c]	7	30
14	BIF_00823	ADC85571	Cell division ATP-binding protein	47 (45)	$3.5e^{-02}$	16	6	30
15	BIF_01972	ADC85645	Protein Translation Elongation Factor Tu	147 (40)	$3.2e^{-12}$	46	14	41
16	BIF_00999	ADC84870	Hypothetical protein	54 (37)	$6.7e^{-03}$	19	8	44
17	BIF_00348	ADC85723	Glutamine synthetase	48 (42)	$2.6e^{-02}$	27	7	72
18	BIF_00608	ADC84634	1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase	46 (36)	$4.3e^{-02}$	23	5	45
19	BIF_00998	ADC84872	Collagen adhesion protein	61 (39)	$1.3e^{-03}$	15	10	47
20	BIF_00315	ADC84590	FtsI; Cell division protein	212 (45[c])	$1.3e^{-16}$	38	19	47
20	BIF_01492	ADC84894	Glycosyltransferase	53 (45[c])	$3.3e^{-02}$	24	8	47
21	BIF_00469	ADC84507	Maltose/maltodextrin-binding protein	89 (29)	1.9e-06	39	8	29
22	BIF_01197	ADC86032	Enolase	193 (40)	$8.1e^{-17}$	48	19	51
23	BIF_00999	ADC84870	Hypothetical protein	49 (40)	$2.3e^{-02}$	17	7	49
24	BIF_00277	ADC85866	Phosphoglycerate kinase	63 (45)	$7.4e^{-03}$	19	7	28
25	BIF_01620	ADC84948	Maltose/maltodextrin-binding protein	74 (35)	$6.6e^{-05}$	21	6	38
26	BIF_00825	ADC85573	Immunogenic secreted protein	89 (41)	$1.9e^{-06}$	29	12	46
26	BIF_01863	ADC84622	Hypothetical protein	61 (41)	$1.2e^{-03}$	35	6	46
26	BIF_00758	ADC85937	Hypothetical exported protein	51 (41)	$1.2e^{-02}$	18	8	46
27	BIF_01972	ADC85645	Protein Translation Elongation Factor Tu	93 (34)	$7.7e^{-07}$	39	11	48
27	BIF_00825	ADC85573	Immunogenic secreted protein	87 (34)	3.1^{-06}	26	12	48
28	BIF_00999	ADC84870	Hypothetical protein	68 (31)	$2.6e^{-04}$	23	10	46
29	N.S.[b]							
30	BIF_01597	ADC84973	Oligo-1,6-glucosidase	61 (37)	$1.2e^{-03}$	16	7	23

30	BIF_01172	ADC84811	Thioredoxin	53 (37)	$7.4e^{-03}$	21	5	23
31	BIF_00287	ADC85858	Transaldolase	60 (44)	$1.6e^{-03}$	24	8	63
32	N.S.							
33	BIF_00998	ADC84872	Collagen adhesion protein	60 (40)	$1.5e^{-03}$	19	6	68
34	N.S.							
35	BIF_01087	ADC86005	Manganese-binding protein	85 (38)	$5.3e^{-06}$	39	11	49
35	BIF_01173	ADC84812	Hypothetical protein	70 (38)	$1.8e^{-04}$	31	10	49
36	BIF_00066	ADC86035	Alkanesulfonates transport ATP-binding protein	60 (42)	$1.5e^{-03}$	19	6	34
37	N.S.							
38	BIF_01173	ADC84812	Hypothetical protein	73 (33)	$7.9e^{-05}$	32	12	65
38	BIF_00066	ADC86035	Alkanesulfonates transport ATP-binding protein	64 (33)	$6.4e^{-04}$	35	10	65
39	BIF_01173	ADC84812	Hypothetical protein	115 (32)	$5.1e^{-09}$	37	13	51
40	BIF_00679	ADC84889	Inosine-uridine preferring nucleoside hydrolase	50 (42)	$2.9e^{-03}$	24	6	43
41	BIF_01290	ADC84758	Membrane-bound transglycosylase	83 (33)	$8.7e^{-06}$	28	9	49
42	BIF_00207	ADC85878	Choloylglycine hydrolase	119 (31)	$2.0e^{-09}$	42	10	31
43	BIF_00973	ADC84814	Carbamoyl-phosphate synthase large chain	89 (35)	$2.3e^{-06}$	31	10	57
44	BIF_01290	ADC84758	Membrane-bound transglycosylase	83 (36)	$8.7e^{-06}$	28	9	48
45	BIF_00825	ADC85573	Immunogenic secreted protein	80 (33)	$1.8e^{-05}$	23	10	38
46	BIF_01234	ADC85371	Manganese-binding protein	71 (30)	$1.4e^{-04}$	39	7	36
47	BIF_01290	ADC84758	Membrane-bound transglycosylase	169 (43)	$2.0e^{-14}$	27	10	14
48	BIF_01914	ADC85476	Cell wall-associated hydrolase	46 (39)	$4.1e^{-02}$	19	4	29
49	BIF_00936	ADC85685	Two component system histidine kinase	55 (44)	$4.9e^{-03}$	26	9	94
50	BIF_00888	ADC84510	*O*-Acetyltransferase (cell wall biosynthesis)	47 (37)	$3.3e^{-02}$	19	7	36
51	BIF_00825	ADC85573	Immunogenic secreted protein	56 (38)	$4.0e^{-03}$	17	7	27
52	BIF_00825	ADC85573	Immunogenic secreted protein	58 (37)	$2.5e^{-03}$	14[c]	6	22
53	BIF_01084	ADC84928	Tetrapyrrole (Corrin/Porphyrin) methylase family protein	80 (31)	$1.8e^{-05}$	26	6	12
54	N.S.							
55	BIF_01914	ADC85476	Cell wall-associated hydrolase	75 (34)	$5.6e^{-05}$	27	6	37
56	N.S.							
57	N.S.							
58	N.S.							
59	BIF_02083	ADC86083	Smf protein	52 (34)	1.1^{-02}	19	4	17
60	N.S.							
61	BIF_00825	ADC85573	Immunogenic secreted protein	54 (32)	$7.0e^{-03}$	14	6	16
62	N.S.							
63	N.S.							
64	BIF_01523	ADC84776	UDP-glucose 6-dehydrogenase	50 (35)	$1.6e^{-02}$	30	8	75
65	BIF_01997	ADC84674	Isoleucyl-tRNA synthetase	52 (39)	$1.1e^{-02}$	19	16	97
66	BIF_01565	ADC85095	Hypothetical protein ylxX/ylxW	58 (40)	$2.8e^{-03}$	19	6	36
67	BIF_00497	ADC85881	Peroxiredoxin	74 (42)	$5.9e^{-05}$	37	7	39
68	BIF_01115	ADC84845	Inorganic pyrophosphatase	127 (32)	$3.2e^{-10}$	40	8	20
69	BIF_01657	ADC85069	Iron-binding ferritin-like antioxidant protein	107 (37)	$3.2e^{-08}$	58	10	57
70	BIF_02033	ADC85125	Hypothetical protein	46 (38)	$4.0e^{-02}$	24	5	63

71	N.S.							
71	N.S.							
72	BIF_00860	ADC84647	Diaminopimelate epimerase	52 (40)	$1.1e^{-02}$	19	6	93
73	BIF_02065	ADC85691	Aspartyl-tRNA synthetase	62 (39)	$9.7e^{-04}$	16	11	49
74	BIF_01746	ADC86104	Peptidyl-prolyl cis-trans isomerase	55 (40)	$5.0e^{-03}$	36	4	43
75	BIF_00682	ADC85747	Arginine repressor	54 (32)	$6.3e^{-03}$	46	4	25
76	BIF_01398	ADC85475	Immunogenic secreted protein	72 (41)	$1.1e^{-04}$	32	7	44
77	BIF_01067	ADC85105	Hypothetical signal transduction protein	112 (37)	$4.0e^{-10}$	43	12	54
77	BIF_01394	ADC85165	Hypothetical protein	62 (37)	$1.6e^{-03}$	54	6	84
78	BIF_00400	ADC85681	60 kDa chaperonin GROEL	84 (30)	$5.9e^{-06}$	14[c]	7	17
79	BIF_01290	ADC84758	Membrane-bound transglycosylase	79 (34)	$3.8e^{-05}$	21	7	24
80	BIF_01290	ADC84758	Membrane-bound transglycosylase	69 (40)	$1.1e^{-05}$	33	10	86
81	BIF_01398	ADC85475	Immunogenic secreted protein	92 (36)	$1.1e^{-05}$	30	7	33
82	N.S.							
82	N.S.							
82	N.S.							
83	BIF_01398	ADC85475	Immunogenic secreted protein	79 (38)	$2.0e^{-05}$	42	9	46
84	BIF_01290	ADC84758	Membrane-bound transglycosylase	49 (26)	$1.1e^{-04}$	35	10	84
85	BIF_01915	ADC85796	Uridylate kinase	82 (36)	$1.0e^{-02}$	16	4	14
86	BIF_01398	ADC85475	Immunogenic secreted protein	121 (34)	$6.4e^{-09}$	46	11	34
87	BIF_01398	ADC85475	Immunogenic secreted protein	84 (35)	$6.3e^{-06}$	42	10	38
88	BIF_01398	ADC85475	Immunogenic secreted protein	104 (41)	$2.6e^{-07}$	49	12	61
89	N.S.							
90	N.S.							
91	N.S.							
92	N.S.							
93	BIF_00325	ADC85424	Nucleoside-diphosphate kinase	79 (38)	$2.2e^{-05}$	44	8	69
94	BIF_01641	ADC84909	UDP-galactofuranosyltransferase	47 (40)	3.5^{-02}	16	7	49
95	BIF_00644	ADC85907	Thioredoxin peroxidase	205 (35)	$5.1e^{-19}$	79	14	53
96	BIF_01290	ADC84758	Membrane-bound transglycosylase	83 (34)	$8.5e^{-06}$	20	6	20
97	BIF_00414	ADC85770	Hypothetical cytosolic protein	68 (40)	$2.5e^{-04}$	38	5	40
98	BIF_01914	ADC85476	Cell wall-associated hydrolase	50 (42)	$5.0e^{-03}$	16	5	36
98	BIF_00377	ADC85900	Probable glutamate-ammonia-ligase adenylyltransferase	46 (42)	$4.4e^{-02}$	7[c]	5	14
99	N.S.							
100	BIF_02023	ADC84801	Transposase	47 (37)	$3.5e^{-02}$	25	7	64
101	N.S.							
102	BIF_00377	ADC85900	Probable glutamate-ammonia-ligase adenylyltransferase	54 (36)	$6.3e^{-03}$	19	15	93
102	BIF_00678	ADC85385	UDP-*N*-acetylenolpyruvoylglucos amine reductase	54 (36)	$6.9e^{-03}$	33	10	93
103	N.S.							
104	N.S.							
105	BIF_01398	ADC85475	Immunogenic secreted protein	84 (38)	$3.2e^{-05}$	34	9	33
106	N.S.							
107	BIF_02023	ADC84801	Transposase	53 (37)	$7.5e^{-03}$	27	9	70
108	N.S.							
109	BIF_01398	ADC85475	Immunogenic secreted protein	75 (38)	$5.7e^{-05}$	30	7	15

110	N.S.							
111	BIF_01290	ADC84758	Membrane-bound transglycosylase	83 (33)	$8.3e^{-06}$	16	6	14
112	N.S.							
112	BIF_00066	ADC86035	Alkanesulfonates transport ATP-binding protein	49 (40)	$2.1e^{-02}$	16	4	18
113	BIF_00377	ADC85900	Probable glutamate-ammonia-ligase adenylyltransferase	47 (41)	$3.3e^{-02}$	15[c]	12	82
114	BIF_01290	ADC84758	Membrane-bound transglycosylase	48 (32)	$2.6e^{-02}$	14[c]	4	14
115	BIF_01012	ADC84864	23S rRNA methyltransferase	50 (43)	$1.5e^{-02}$	28	6	62
116	BIF_01289	ADC84757	Thioredoxin	129 (43)	$1.0e^{-10}$	66	11	59
117	BIF_00278	ADC85867	Hypothetical cytosolic protein	46 (35)	$4.0e^{-02}$	35	8	73
118	BIF_02020	ADC85288	RecG	53 (43)	$7.5e^{-03}$	36	5	95
119	N.S.							
120	BIF_00644	ADC85907	Thioredoxin peroxidase	68 (42)	$5.9e^{-05}$	56	7	56
121	N.S.							
122	BIF_01132	ADC85609	Glycerol dehydrogenase	52 (36)	$1.0e^{-02}$	24	6	59
123	BIF_01054	ADC84884	SSU ribosomal protein S6P	78 (44)	$1.6e^{-05}$	67	6	44
124	BIF_00675	ADC85383	10 kDa chaperonin GROES	112 (41)	$1.0e^{-08}$	78	8	87
X1	BIF_00737	ADC84524	Oligopeptide-binding protein	153 (41)	$8.1e^{-13}$	30	5	11
X2	BIF_00212	ADC85538	Sugar-binding protein	178 (28)	$2.6e^{-15}$	37	14	26

[a] Peptide mass fingerprinting (PMF) score obtained using the protein sequence database program Mascot (http://www.matrixscience.com) with the genome sequence of *Bifidobacterium animalis* subsp. *lactis* BB-12 as taxonomy entry. Scores > 45 are statistically significant, and the scores obtained for identical searches against a decoyed database are designated in parenthesis. Spot numbers refer to the spots designated in Figure 1. Spots X1 and X2 were identified in gels derived cultures grown on xylo-oligosaccharides. [b] No statistically significant MS hit obtained. [c] Sequence coverage ≤ 15.