A Mechanistic Study of the Fermentation of β-glucans from Different Sources by Bifidobacteria

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Abstract

β-Glucans are a kind of non-digestible carbohydrate (NDC) that are known for their benefits for human gut health, but there are very few studies on their fermentability by human colon microbiota. In this study four β-glucans were selected for *in vitro* fermentation by three bifidobacteria. The β-glucans included those from a seaweed called *Laminaria digitata* (laminarin), barley, a bacterium called *Alcaligenes faecalis* (curdlan), and a mushroom sclerotia from *Pleurotus tuber-regium*. Inulin from Dahlia tubers was used as control.

The content of β-glucan in the NDCs prepared from the mushroom sclerotium of *Pleurotus tuber-regium* was 80.8 % with proteins less than 1.0 %, while that of curdlan, barley and laminarin all have more than 95 % β-glucan. All the β-glucans contained almost purely glucose as their sugar components with only trace amount of mannose (< 2%) being found in laminarin. β-glucan from barley had a MW of 590 kDa and a linear chain with mixed 1→3 and 1→4 β-linkages in the ratio of 1:3. Curdlan had a β-(1→3) linked unbranched linear chain with a MW of 10 to 30 kDa. Laminarin had a β-(1→3) linked backbone with β-(1→6) branches, having a MW of 6 kDa. β-Glucan from mushroom sclerotia had a highly branched main chain with mixed glycosidic 1→3, 1→4 and 1→6 β-linkages with a MW of 96 kDa.

Batch systems of *in vitro* fermentation of individual NDCs by *B.longum* subsp. *infantis* (*B. infantis*), *B. longum* and *B. adolescentis* were carried out for 24 h under anaerobic condition. All the systems showed a significant drop ($p< 0.05$) of at least 0.5 units in their pH values. The populations of *B. infantis* increased by 3 $\log_{10}$ CFU

on all the NDCs while those of *B. longum* and *B. adolescentis* increased by about 1 to 1.5 $\log_{10}$ CFU and 2 to 2.3 $\log_{10}$ CFU, respectively. Utilization of the NDCs by the bifidobacteria evaluated by organic matter disappearance ranged from 4.52 % in barley to 41.3 % in inulin. The total short chain fatty acid (SCFA) produced by *B. infantis* was higher than that in *B. longum* and *B. adolescentis* for all the β-glucans. The SCFA profile of inulin and all β-glucans produced by all the bifidobacteria was dominated by acetate (96%). The ratio of acetic : propionic : butyric acid in the SCFA profile of the fermentation of all the β-glucans by *B. infantis* was 8:1:1, which was very different from that of *B. longum* and *B. adolescentis*.

Based on the *in vitro* fermentation results, *B. infantis* was selected for a mechanistic study on the fermentation of the β-glucans from different sources by proteomic and molecular biology approaches. In the proteomic study, the gels of the two-dimensional difference gel electrophoresis (2D-DIGE) containing the total proteins from the *B. infantis* cells fermented with β-glucans from barley, seaweed and mushroom sclerotia were compared with each other to isolate the differentially expressed protein spots. In all the comparisons, a total number of 198 protein spots were identified based on their mass spectra. These proteins were classified according to their functional annotation, including ABC transporters, phosphotransferase system (PTS), transketolase and others. Several genes encoding the proteins that probably play a role in the transport and degradation of β-glucans including the ABC transporter gene, PTS gene and membrane protein gene underwent real time RT-PCR for transcriptional analysis. Hydrolytic enzyme activity assay showed that

intracellular β-1, 3 glucanase activity was present when *B. infantis* was incubated with β-glucans from seaweed and mushroom.

Based on the above results, a model for β-glucan catabolism in *B. infantis* was proposed. The β-glucan molecules might be captured and imported inside the bacterial cells either by ABC transporters or PTS. They were then subjected to hydrolysis by glucan β-1, 3 glucosidase. The released glucose molecules were readily incorporated into the central fermentation pathway, the 'bifid shunt', in which the hydrolyzed residues were further degraded or exported. This study has deepened our understanding on the fermentation of β-glucans by bifidobacteria and demonstrated the potential of β-glucans to be used as a novel prebiotic.

摘要

葡聚糖是非消化性糖類的一種，對於人體腸道健康具有多種益處，然而目前關於其被人體腸道菌群發酵特性的研究非常之有限。本課題利用三種雙歧桿菌對四種不同來源之葡聚糖進行體外發酵研究，包括海藻，大麥，細菌以及蘑菇菌核來源之葡聚糖，同時以菊粉多糖作為陽性對照。所有多糖均進行單糖組成及結構分析。蘑菇來源多糖中葡聚糖含量為 80.8 %，其餘三種多糖葡聚糖含量均高於 95%。所有多糖都以葡萄糖為基本單糖成份，只有海藻來源者含有微量甘露糖(< 2%)。大麥多糖分子量約為 590 kDa，線形多糖含 β-1,3 和 β-1,4 連接的比例為 1:3。細菌多糖為 β-1,3 連接的無分支多糖，已報導分子量為 10 至 30 kDa。海藻多糖分子量接近 6 kDa，由 β-1,3 連接主糖鏈并由 β-1,6 連接分支結構。蘑菇多糖由 β-1,3，β-1,4 和 β-1,6 連接構成高度分支結構，分子量 96 kDa。

所有多糖分別被三種雙歧桿菌無氧條件下發酵培養 24 小時，所有系統均呈現 pH 降低至少 0.5 單位。嬰兒雙歧在所有多糖中菌落數目呈現三倍指數增長，長雙歧桿菌增長 1 至 1.5 倍，青春雙歧增長 2 至 2.3 倍。所有多糖有機物消耗率在 4.52 %（大麥）到 41.3 %（菊粉）之間。三種細菌發酵菊粉產生主要為乙酸(96%)。嬰兒雙歧桿菌產生短鏈脂肪酸總量高於其它二者且發酵所有多糖產生乙酸，丙酸，丁酸的比例為 8:1:1，區別于其它兩者。

基於發酵實驗結果，嬰兒雙歧桿菌被選作進一步發酵葡聚糖機理研究。在蛋白質組學實驗中，嬰兒雙歧桿菌發酵不同來源多糖后，體內所有蛋白質被提取出來作雙向電泳處理，不同處理的電泳膠彼此對比，目的是找出不同處理之間表達量發燒變化的蛋白質點。在所有對比之中，總共 198 被成功鑒定出質譜結果并被分類。幾個對於發酵葡聚糖起重要作用的蛋白質被挑選出來，包括轉運蛋白，膜蛋白等，進行下一步轉錄水平研究。嬰兒雙歧桿菌發酵海藻和蘑菇葡聚糖時產生細胞內 β-1, 3 葡聚糖。

綜合幾種方法獲得的結果，提出嬰兒雙歧桿菌利用葡聚糖的分子模型。發酵液中的葡聚糖分子被細胞壁上的 PTS 或者 ABC 轉運系統捕獲並且吸收至細胞內部，隨即被 β-1, 3 葡聚糖酶降解，釋放出的葡萄糖分子進入碳源發酵途徑，被逐步降解至產生短鏈脂肪酸等代謝產物。本研究意在擴大對雙歧桿菌發酵利用葡聚糖機理的理解，以及證明葡聚糖作為新型益生元的潛力。

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B. infantis	*Bifidobacterium longum* subsp. *infantis*
DP	Degree of polymerization
FOS	Fructooligosaccharides
GIT	Gastrointestinal tract
GOS	Galactooligosaccharides
LAB	Lactic acid bacteria
NDC	Nondigestible carbohydrate
NSP	Nondigestible polysaccharide
OMD	Organic matter disappearance
PC	*Poria cocos*
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
PTR	*Pleurotus tuber-regium*
SCFA	Short-chain fatty acid
2D-DIGE	Two dimensional difference gel electrophoresis
TOS	Transgalactosylatedoligosaccharides
XOS	Xylooligosaccharide

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Chapter 1 Introduction

1.1 Probiotics

Probiotics are living microorganisms, which upon ingestion in sufficient amount can exert health benefits beyond inherent basic nutrition (Guarner and Schaafsma 1998). Actually the recognition of probiotic effects should be dated back to the beginning of 20th century, when Metchnikoff connected the longevity of Bulgarian peasants with the high intake of soured milks (Gibson 2004). The actions through which probiotics exert their health beneficial effects can be classified into three modes (Oelschlaeger 2010) : (a) Enhance the host defence system through modulating the immune system; (b) Modulate the microbial equilibrium in the gastrointestinal tract through direct effect on other microorganisms; and (c) Inactivate and detoxify the harmful microbial metabolites in the host and food ingredients. A potential probiotic candidate is expected to possess several desirable properties so as to exert its beneficial effects. The relevant criteria for potential probiotics are summarized in Table 1.1 (Ouwehand et al. 1999). A probiotic candidate should be originated from human if intended for human use. It should sustain under acid and bile surrounding, which is vital for oral administration, survival through the intestine and retain its metabolic activity. It should adhere to the mucosal surfaces in order to help modulate the immune system, inhibiting pathogen adhesion. The candidate strain should be well characterized and documented safe for

food and clinical uses as well as clinically validated health effects. It should possess good technological properties allowing it to be stable and survive in the product (Ouwehand et al. 1999).

Besides the above properties, some additional features may be needed for specific outcomes. Some probiotic strains could lower serum cholesterol level; produce vitamins; reduce mutagen level; inhibit the activity of potential cariogenic microorganisms; and produce anti-microbial substances. Some probiotic bacteria could catabolize lactose for β-galactosidase deficient subjects. But not all these criteria need to be fulfilled for one potential probiotic candidate. Based on these beneficial effects on human health, many probiotics have been selected to be included in probiotic preparations, most of which are lactic acid bacteria, which belong to the genus of *Lactobacillus* and *Bifidobacterium* (as shown in Table 1.2) (Parvez et al. 2006).

Table 1.1 Selection criteria for potential probiotic microorganism and additional features (Ouwehand et al. 1999)

Probiotic strain properties	**Additional features**
Human origin	↓ Cholesterol metabolism
Acid and bile stability	↑ Bioavailability of vitamins and minerals
Adhesion to mucosal surfaces	↓ Faecal mutagen levels
Safe for food and clinical use	↑ Metabolism of lactose
Clinically validated health effects	↓ Anti-caries activity
Good technical properties	↑ Production of antimicrobial substances

Table 1.2 The most commonly used species of lactic acid bacteria in probiotic preparations

Lactobacillus sp.	*Bifidobacterium* sp.	*Enterococcus* sp.	*Streptococcus* sp.
L. acidopholius	*B. bifidum*	*Ent. Faecalis*	*S. cermoris*
L. casei	*B. adolescentis*	*Ent. faecium*	*S. salivarius*
L. delbrueckii ssp. *bulgaricus*	*B. animalis*		*S. diacetylactis*
L. cellobiosus	*B. infantis*		*S. intermedius*
L. curvatus	*B. thermophilum*		
L. fermentum	*B. longum*		
L. lactis			
L. plantarum			
L. reuteri			
L. brevis			

Adapted from Parvez et al. (2006)

1.1.1 Mechanisms of probiotic action

Figure 1.1 summarizes the effects on the possible health benefits of probiotics. Probiotics can influence the host defence through the innate and the acquired immune system by products such as microbial metabolites, cell wall components, etc. Even dead probiotic bacteria (Zhang, Ohta and Hosono 1990) or probiotic derived components, such as cell wall peptidoglycans (Stewart-Tull 1980), could trigger the immune modulatory effects. Probiotics exert their effects on the immune system in a strain specific manner, but the molecular basis is still unclear (Oelschlaeger 2010).

Probiotics show their direct influences on the other microorganisms in the intestine in different ways. Probiotics are able to produce antimicrobial substances, including lactic acid, hydrogen peroxide, bactreriocins as well as deconjugated bile acids to inhibit the growth of pathogenic microorganisms (Maqueda et al. 2008). Certain nutrient substances, such as iron, in the host are in a limited amount and some probiotic strains, such as *Lactobacillus*, are able to bind to the iron molecule on the cell surface making it unavailable for pathogenic strains (Elli et al. 2000). Probiotic bacteria are able to adhere to the epithelial cells at the same time blocking the adherence of pathogenic ones by the competition for specific receptors or induction of mucin production (Mack et al. 2003).

Some probiotcs may protect the host against toxins expressed from pathogens, such as Shiga toxin from *E. coli*, which is the most important group of bacterial virulence factors. Besides, there are studies on the anti-cancer activity of probiotics.

Probiotics can amplify the immune response to tumor tissue (Hirayama and Rafter 2000), or, on the other way, they can bind harmful compounds to reduce their carcinogenic effect and damage to DNA (Geier, Butler and Howarth 2006; Oelschlaeger 2010). A detailed mechanistic analysis of these beneficial effects of probiotics on the molecular level can provide information for the design of recombinant probiotic strain possessing specific desired functions.

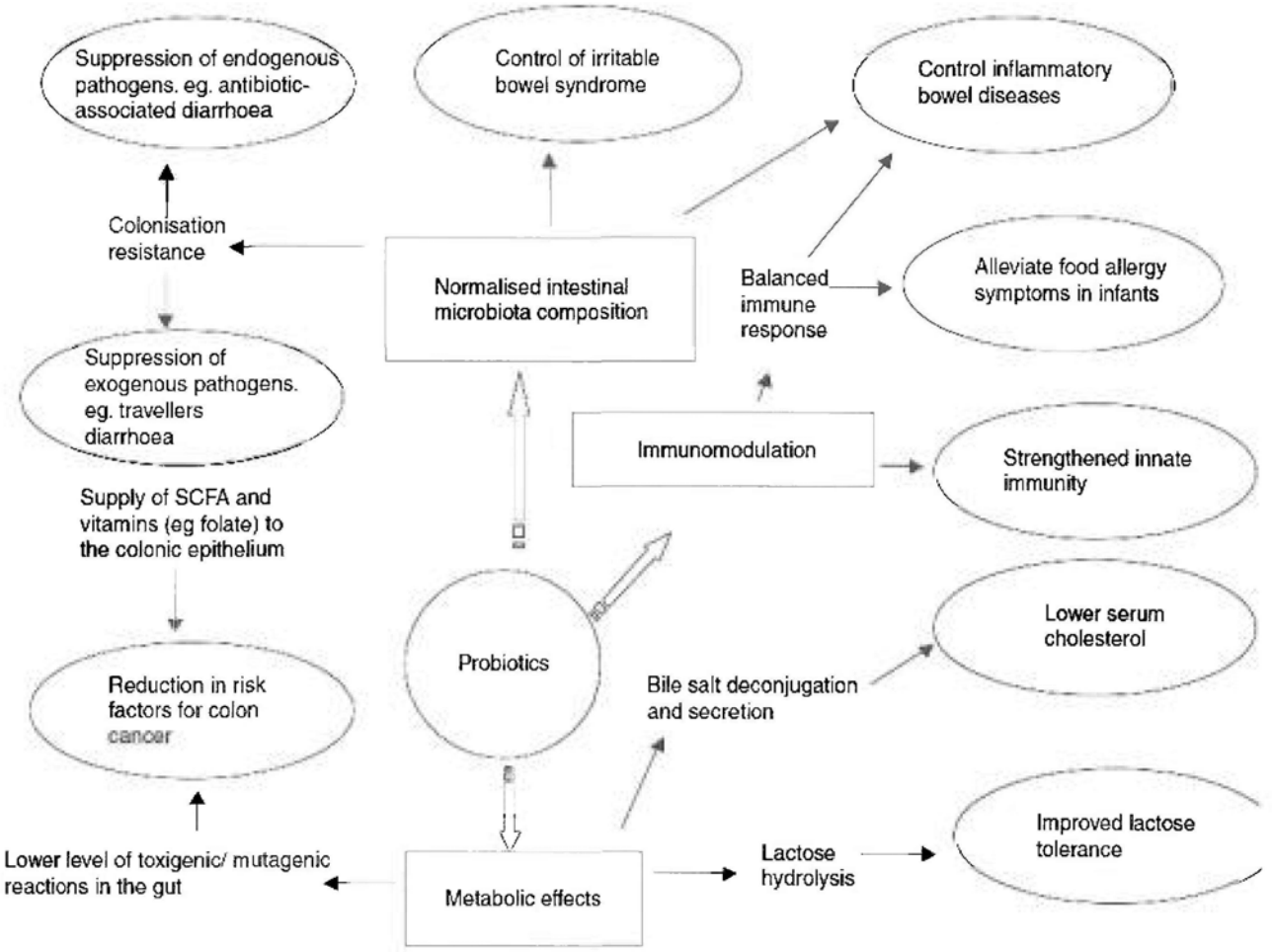

Fig. 1.1 Various health benefits from probiotics consumption
Adapted from Parvez et al. (2006)

1.1.2 Classification of *Bifidobacterium*

Bifidobacteria, accounting for nearly 95 % of the gut microflora of breast-fed babies and up to 3 % of the total colonic microflora in adults, are thought to play an important role in human health (Parche et al. 2007). Tissier (1900) isolated one *Bifidobacterium* species for the first time from the breast-fed infant faeces, and named it *Bacillus bifidus communis* (Tisser, 1900). Bifidobacteria are Gram-positive, non-spore forming, non-motile anaerobic bacteria, belonging to the high G+C content group. Bifidobacteria can produce lactic acid as one of the fermentation end products and are often considered as heterofermentative lactic acid bacteria (LAB). Like most neighbors in the colon, bifidobacteria are able to ferment the carbohydrates in the colon, and their substrates include various complex carbon sources, such as xylo-oligosaccharides, pectin, galactooligosaccharides, soy bean oligosaccharides and other plant-derived oligosaccharides, with differences between strains (De Vrese and Schrezenmeir 2008).

As summarized in Table 1.3, there are 39 species that have been characterized in the *Bifidobacteirum* genus. There are 24 different strains that have been subjected to sequence analysis and their general genome features are summarized in Table 1.4 (Pokusaeva, Fitzgerald and van Sinderen 2011b). The first complete genome sequence of *B. longum* subsp. *longum* NCC2705 was released in 2002 (Schell et al. 2002). Another 10 bifidobacterial genomes have been fully sequenced and annotated (Table 1.4), including *B. longum* (DJO10A, JDM301), *B. infantis* ATCC15697, *B. adolescentis* ATCC15703, four *B. animalis subsp. lactis* (DSM10140, AD011, B1-04

and Bb12), *B. bifidum* PRL2010 and *B. dentium* Bd1. An additional 13 genome drafts are currently present in the database of the National Center for Biotechnology Information (NCBI). The rapid growth in the genome study for bifidobacteria is a clear reflection of the growing scientific interest in this particular group aiming at the genetic basis for their health benefits, adaptation to colonization and persistence in the human gastrointestinal tract (GIT) (Pokusaeva, Fitzgerald and van Sinderen 2011b).

1.1.3 Bifidobacterial carbohydrate metabolism

The predicted proteins (enzymes) from the genome information provide the molecular basis for the explanation that these bifidobacteria are well equipped for colonization in the human colon (Table 1.5) (Pokusaeva, Fitzgerald and van Sinderen 2011b). The ability of bifidobacteria to degrade complex carbohydrates in the colon has already been well established, and the presence of genes that encode various carbohydrate-modifying enzymes reflects their adaptation to the human gastrointestinal tract environment (Walker, Cerdeño-Tárraga and Bentley 2006). More than 8 % of the genes in a given bifidobacterial genome is thought to be involved in carbohydrate metabolism and nearly half of these genes, that is about 5 % of the total bifidobacterial genes, functions in carbohydrate uptake. This phenomenon indicates their acquired adaptations to allow the bifidobacteria to exploit a rich repertoire of indigestible components of the human diet (Ventura et al. 2009) (Pokusaeva, Fitzgerald and van Sinderen 2011b).

Table 1.3 Known *Bifidobacterium* species

Bifidobacterial species	Animal origin
B. actinicoloniformis	Bumblebee intestine
B. adolescentis	Adult intestine
B. angulatum	Human faeces, sewage
B. animalis subsp. *animalis*	Chicken, rat, rabbit and calf faeces; river
subsp. *lactis*	Fermented milk
B. asteroides	Hindgut of honeybee
B. bifidum	Adult intestine, human faeces, vagina
B. bohemicus	Bumblebee intestine
B. bombi	Bumblebee intestine
B. boum	Bovine rumen; piglet faeces
B. breve	Infant intestine, faeces, vagina
B. catenulatum	Child and adult intestine, vagina
B. choerinum	Pig faeces
B. coryneforme	Hindgut of honeybee
B. crudilactis	Raw milk and raw milk cheese
B. cuniculi	Rabbit faeces
B. dentium	Human dental caries, faeces, human vagina
B. gallicum	Adult intestine
B. gallinarum	Chicken cecum
B. indicum	Bees; river
B. longum subsp. *infantis*	Infant intestine, vagina
subsp. *suis*	Feces of piglet
subsp. *longum*	Child and adult intestine, vagina
B. magnum	Rabbit faeces; sewage
B. merycicum	Rumen
B. minimum	Sewage
B. mongoliense	Fermented mare's milk product
B. pseudocatenulatum	Child faeces
B. pseudolongum subsp. *globosum*	Pig, chicken, calf and rat faeces, rumen
subsp. *pseudolongum*	Pig, chicken, calf and rat faeces, rumen
B. psychraerophylum	Porcine caecum
B. pullorum	Chicken faeces
B. ruminantium	Rumen
B. saeculare	Rabbit faeces
B. scardovii	Adult faeces
B. subtile	Sewage
B. thermophilum	Pig chicken and calf faeces, bovine rumen
B. thermacidophilum	Anaerobic digester
B. tsurumiense	Hamster dental plaque

Adapted from Pokusaeva, Fitzgerald and van Sinderen (2011b)

Table 1.4 General features of completely sequenced genomes in *Bifidobacterium*

Species	Genome size (bp)	Genes	Proteins	Source
B. adolescentis ATCC15703	2,089,645	1,701	1,631	Human GIT
B. adolescentis L2-32	2,385,710	2,499	2,428	Infant
B. animalis subsp. *lactis* HN019	1,915,892	1,632	1,578	Infant
B. animalis subsp. *lactis* DSM10140	1,938,483	1,629	1,566	French Yogurt
B. animalis subsp. *lactis* AD011	1,933,695	1,604	1,528	Infant faeces
B. animalis subsp. *lactis* Bl-04	1,938,709	1,631	1,567	Adult faeces
B. animalis subsp. *lactis* Bb12	1,942,198	1,624	NA	Fermented milk
B. angulatum DSM20098	2,007,108	1,811	1,748	Human faeces
B. bifidum NCIMB41171	2,186,140	1,888	1,833	Adult faeces
B. bifidum PRL2010	2,214,650	1,848	NA	Human GIT
B. breve UCC2003	2,422,668	1,868	1,590	Infant faeces
B. breve DSM20213	2,297,799	2,309	2,251	Human faeces
B. dentium ATCC27678	2,642,081	2,500	2,336	Urogenital tract
B. dentium Bd1	~2,600,000	~2,270	NA	Dental caries
B. longum subsp. *longum* DJO10A	2,375,792	2,062	1,990	Human GIT
B. longum supsp. *longum* NCC2705	2,256,640	1,798	1,727	Human GIT
B. longum subsp. *infantis* ATCC15697	2,832,748	2,588	2,416	Infant GIT
B. longum subsp. *longum* JDM301	2,477,838	2,035	1,958	Human faeces
B. longum subsp. *infantis* ATCC55813	2,372,858	2,171	2,109	Infant GIT
B. longum subsp. *infantis* CCUG52486	2,453,376	2,296	2,240	Infant GIT
B. pseudocatenulatum DSM20438	2,304,808	2,220	2,151	Human faeces
B. catenulatum DSM16992	2,058,429	2,011	1,950	Human faeces
B. gallicum DSM20093	2,016,380	2,045	1,983	Human faeces

Adapted from Pokusaeva, Fitzgerald and van Sinderen (2011b)

Table 1.5 Putative carbohydrate-modifying enzymes identified in the genomes of some bifidobacterial species

EC number	Enzyme name	*B. longum* subsp. *longum* ATCC15697	*B. longum* subsp. *Infantis* ATCC55813	*B. longum* subsp. *infantis* CCUG52486	*B. longum* subsp. *longum* DJO10A	*B. longum* subsp. *longum* NCC2705
Hexosyltraspherases						
EC:2.4.1.1	Phosphorylase	1	1	1	1	1
EC:2.4.1.7	Sucrose phosphorylase	1	1	1		1
EC:2.4.1.18	1,4-alpha-glucan branching enzyme	1	1	1	1	1
EC:2.4.1.25	4-alpha-glucanotransferase	2	2	2	2	2
EC:2.4.1.23	Kojibijose phosphorylase	—	—	—	—	—
Phosphotrasferases						
EC:2.7.1.12	Gluconokinase	—	1	1	1	1
EC:2.7.1.15	Ribokinase	2	4	2	2	2
EC:2.7.1.17	Xylulokinase	1	2	2	2	2
EC:2.7.1.31	Glycerate kinase	1	1	1	1	1
EC:2.7.1.4	Fructokinase	1	2	1	1	1
EC:2.7.1.6	Galactokinase	1	1	1	1	1
EC:2.7.6.1	Ribose-phosphate diphosphokinase	2	2	2	2	2
Glycosyl hydrolases						
EC:3.2.1.1	Alpha-amylase	1	—	—	—	—
EC:3.2.1.10	Oligo-1,6-glucosidase	1	—	2	1	1
EC:3.2.1.14	Chitinase	—	—	—	—	1
EC:3.2.1.18	Exo-alpha-sialidase	2	—	—	—	—

Table 1.5 (continued)

EC number	Enzyme name	*B. longum* subsp. *longum* ATCC15697	*B. longum* subsp. *infantis* ATCC55813	*B. longum* subsp. *infantis* CCUG52486	*B. longum* subsp. *longum* DJO10A	*B. longum* subsp. *longum* NCC2705
EC:3.2.1.20	Alpha-glucosidase	1	2	1	2	1
EC:3.2.1.21	Beta-glucosidase	2	3	2	2	1
EC:3.2.1.22	Alpha-galactosidase	1	1	1	1	1
EC:3.2.1.23	Beta-galactosidase	4	4	3	3	3
EC:3.2.1.24	Alpha-mannosidase	2	2	—	3	3
EC:3.2.1.25	Beta-mannosidase	—	—	—	—	—
EC:3.2.1.26	Beta-fructofuranosidase	1	1	1	1	1
EC:3.2.1.31	Beta-glucuronidase	—	—	1	1	—
EC:3.2.1.35	Hyaluronoglucosaminidase	—	—	—	—	—
EC:3.2.1.37	Xylan-1,4-beta-xylosidase	—	—	1	1	—
EC:3.2.1.4	Cellulase	—	—	—	—	—
EC:3.2.1.41	Pullulunase	—	—	—	—	—
EC:3.2.1.45	Glucosylceramidase	—	—	—	—	—
EC:3.2.1.51	Alpha-L-fucosidase	3	—	—	—	—
EC:3.2.1.52	Beta-*N*-acetylhexosaminidase	4	2	2	2	2
EC:3.2.1.54	Cyclomaltodextrinase	1	1	1	1	1
EC:3.2.1.55	Alpha-*N*-arabinofuranosidase	—	3	2	3	3
EC:3.2.1.78	Mannan endo-1,4-beta-mannosidase	—	—	—	—	—
EC:3.2.1.86	6-phospho-beta-glucosidase	—	—	—	—	—
EC:3.2.1.89	Arabinogalactan-endo-1,4-beta-galactosidase	—	—	—	1	1
EC:3.2.1.93	Trehalose-6-phosphate hydrolase	—	—	—	—	1

Table 1.5 (continued)

EC number	Enzyme name	*B. longum* subsp. *longum* ATCC15697	*B. longum* subsp. *infantis* ATCC55813	*B. longum* subsp. *infantis* CCUG52486	*B. longum* subsp. *longum* DJO10A	*B. longum* subsp. *longum* NCC2705
EC:3.2.1.96	Mannosyl-glycoprotein endo-beta-*N*-acetylglucosaminidase	—	—	—	1	1
	Isomerase					
EC:5.3.1.1	Triosephosphate isomerase	1	1	1	1	1
EC:5.3.1.4	L-arabinose isomerase	—	1	1	1	1
EC:5.3.1.5	Xylose isomerase	—	1	1	1	1
EC:5.3.1.6	Ribose-5-phosphate isomerase	1	1	1	1	1
EC:5.3.1.9	Glucose-6-phosphate isomerase	1	1	1	1	1

Numbers in the table indicate the number of proteins in the genome belong to the enzyme group.

Adapted from Pokusaeva, Fitzgerald and van Sinderen (2011b)

Bifidobacteria degrade hexose sugars through the unique metabolic pathway called "bifid shunt", in which fructose-6-phosphoketolase plays a central role and this enzyme could be used as a taxonomic marker for the group (De Vries, Gerbrandy and Stouthamer 1967). Many additional enzymes are needed to work cooperatively to channel sugar molecules from various sources into this particular 'bifid shunt' pathway (Fig. 1.2) (Pokusaeva, Fitzgerald and van Sinderen 2011b). Bifidobacteria produce 2.5 moles of ATP molecules from 1 mole of glucose substrate, with 1.5 mole of acetate and 1 mole of lactic acid. This yield is more efficient than that of the homo- as well as hetero-fermentative ones of lactic acid bacteria (LAB), as the homofermentative group produces 2 moles of ATP and 2 moles of lactic acid from 1 mole of glucose while the heterofermentative LAB produces 1 mole of lactic acid and 1 mole of ATP from 1 mole of glucose. Carbohydrate substrate preference varies among bifidobacterial strains, which is controlled by catabolite regulatory mechanism through synthesis of hydrolytic enzymes and transporter proteins as well as their physiological activities (Macfarlane, Steed and Macfarlane 2008). When grown on various mixtures of arabinose, mannose, galactose, glucose and xylose, *B. longum* co-uitilized glucose and xylose with inhibition of galactose uptake. *B. adolescentis* used glucose and galactose simultaneously with repression of mannose and arabinose. *B. infantis* preferred glucose and galactose whereas *B. bifidum* showed sequential sugar uptake (galactose > glucose > xylose) (Degnan and Macfarlane 1991).

Non-digestible carbohydrates (NDCs) in the colon could be modified by

different enzymes, including phosphotransferase, hydrolase, isomerase and etc., yielding different products. For bifidobacteria, glycoside hydrolases may be the most important group for their adaptation, colonization and competition in the human colon. Most of the early work on isolation and characterization of the carbohydrases from bifidobacteria was finished by Japanese researchers in the 1980s (Kenji et al. 1986). The glycoside hydrolases characterized so far are summarized in Table 1.6 (Van Den Broek et al. 2008). Take β-galactosidase as one example. It is universally distributed in *B. infantis* strains, which is one major species in the colonic microbiota of breast-fed infants, and the galactose is one important component of the oligosaccharides in human milk. The presence of β-galactosidase in *B. infantis* is a clear demonstration of the adaptation of bifidobacteria to their colonization niche (Sela et al. 2008).

Mechanistic models have been proposed to explain the utilization of NDCs at molecular level by colonic bacteria. In *B. longum* biotype *longum* genome, there are a variety of glycanases, among which is a multi-domain glycanase, which includes one transmembrane domain for anchoring in the cell membrane and two carbohydrate binding modules as a catalytic domain to capture NDC substrates such as xylan oligosaccharide (Figure 1.3) (Van Den Broek et al. 2008). Generally speaking, colonic bacteria such as bifidobacteria are capable of capturing and degrading NDCs in their surroundings and transport the polysaccharides or their hydrolytic products into the cells as energy sources.

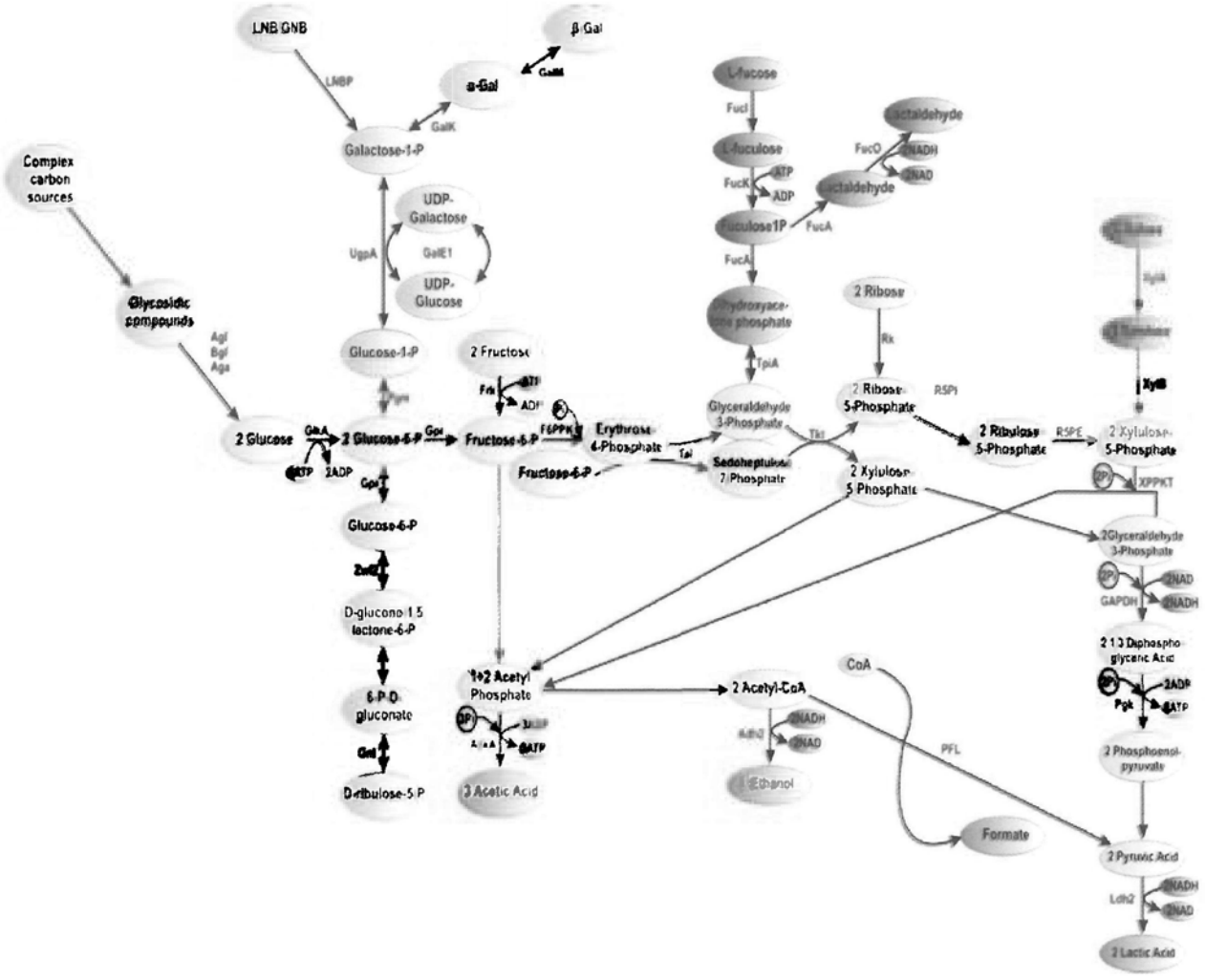

Fig.1.2 Schematic representation of the bifid shunt — hexose sugar degradation pathway in bifidobacteria Adapted from Pokusaeva, Fitzgerald and van Sinderen (2011b). Abbreviations: AckA, acetate kinase; Adh2, aldehyde-alcohol dehydrogenase 2; Aga, a-galactosidase; Agl, a-glucosidase; Bgl, b-glucosidase; GalE1, UDP-glucose 4-epimerase; GalK, galactokinase; GalM, galactose mutarotase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase C; GlkA, glucokinase; Gnt, 6-phosphogluconate dehydrogenase; Gpi, glucose 6-phosphate isomerase; Frk, fruktokinase; F6PPK, fructose-6-phosphoketolase; FucI, L-fucose isomerase; FucK, L-fuculose kinase; FucA, L-fuculose-1P aldolase;FucO, lactaldehyde reductase; Ldh2, lactate dehydrogenase; LNBP, lacto-N-biose phosphorylase;Pgk, phosphoglyceric kinase; Pgm, phosphoglucomutase; Pfl, formate acetyltransferase; Rk, ribokinase; R5PI, ribose-5-phosphate isomerase; R5PE, ribulose-5-phosphate epimerase; Tal, transaldolase; Tkt, transketolase; TpiA, triosephosphate isomerase;UgpA, UTP-glucose-1-phosphate uridylyltransferase; XPPKT, xylulose-5-phosphate/fructose-6-phosphate phosphoketolase; XylA, xylose isomerase;XylB, xylulose kinase; Zwf2, glucose-6-phosphate 1-dehydrogenase; Pi, phosphate

Table 1.6 Glycoside hydrolases from bifidobacteria

Species/strain	Enzyme	Molecular Mass(kDa)	pI	GH family
B. adolescentis				
DSM 20083	D-xylo-isomerase	53	4.3	
	α-glucosidase A	~71	—	13
	α-glucosidase B	73	—	13
	α-galactosidase	83	—	36
	β-galactosidase	89	—	42
	Sucrose phosphorylase	58	—	13
G1	β-fructofuranosidase	74	4.5	
Int-57	α-amylase	66	5.2	13
	β-glucosidase	—	—	
B. longum biotype infantis				
ATCC15697	β-fructofuranosidase	68	4.3	
	α-galactosidase	—	—	36
	β-galactosidase	—	—	42
DSM 20088	β-galactosidase	77	—	42
B. longum biotype longum				
401	β-galactosidase	—	—	42
	lactase	—	—	
B667	α-L-arabinofuranosidase	—	—	51
NCC490	Endo-galactanase	94	—	53
JCM1217	Endo-α-N-acetylgalactos -aminidase	200	—	101
SJ32	Sucrose phosphorylase	56	—	13

Adapted from Van Den Broek et al. (2008)

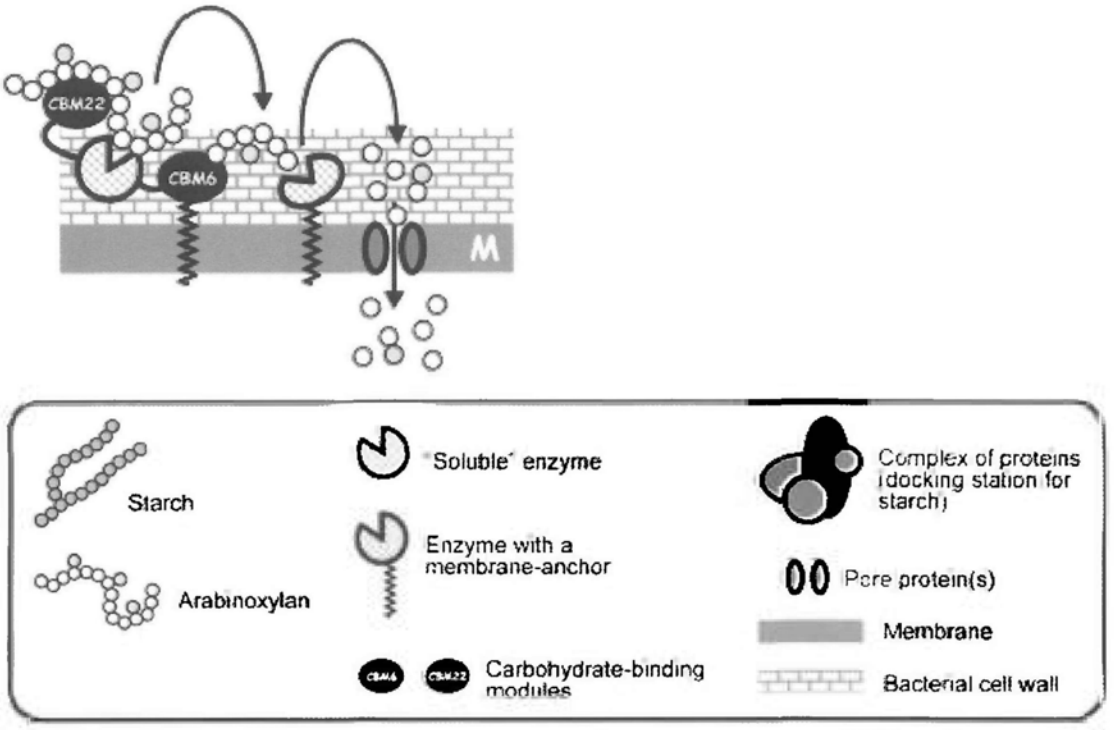

Fig. 1.3 Schematic representation of molecular strategy of *B. longum* for carbohydrate uptake

Adapted from Van Den Broek et al. (2008)

1.2 Prebiotics

1.2.1 Definition and properties of prebiotics

"Prebiotics" are non-digested food ingredients that affect the host by selectively stimulating the growth or activity of one or a number of bacteria in the colon that can improve the host's health (Gibson and Roberfroid 1995). As beneficial food components, prebiotics are expected to work with probiotcs cooperatively to maintain a delicate balance between the gastrointestinal tract and immune system (Parvez et al. 2006).

For a food ingredient, in order to be classified as a prebiotic, it must fulfill the following criteria: (1) resistance to digestion and hydrolysis by host, and to gastrointestinal absorption; (2) fermentation by the microflora in the host gastrointestinal tract (GIT); and (3) stimulation of the growth and/or activity of one or a number of intestinal bacteria in a selective way (Gibson 2004).

According to the criteria above, many food ingredients could be classified as candidate prebiotics, including nondigestible carbohydrates (oligo- and polysaccharides), some peptides and proteins, and certain lipids. For their specific chemical structures, these compounds escape absorption in the upper part of the GIT and hydrolysis by human digestive enzymes. Such compounds could serve as 'colonic foods', which provide substrates for the colonic bacteria, indirectly supplying the host with energy, metabolic products and essential micronutrients (Gibson and Roberfroid 1995). Among these, nondigestible carbohydrates could

fulfill the criteria of prebiotics as mentioned above. Some peptides and proteins, mostly from milk and plants, are known to be nondigestible to some extent, and have some beneficial effects, including facilitating the absorption of cations and stimulating the immune system (Macfarlane and Cummings 1991). But the role of their colonic fermentation on health effects has not been demonstrated, and on the contrary, anaerobic proteolysis may produce some potentially harmly compounds (e.g. ammonia, amines) (Gibson and Roberfroid 1995).

Many compounds could be recognized as 'nondigestible carbohydrates', such as resistant starch, non-starch polysaccharides (plant cell wall polysaccharides, hemicellulose, pectins, gums), and nondigestible oligosaccharides, but not all of them could fullfil the requirement of specific fermentation by colonic microbiota. The major potential prebiotic candidates are listed in Table 1.7 (Macfarlane, Macfarlane and Cummings 2006).

Table 1.7 Properties of the non-digestible oligosaccharides as prebiotic candidates

Name	Composition	Method of manufacture
Inulin	β-(2-1) fructans	Extraction from chicory root
Fructo-oligosaccharides	β-(2-1) fructans	Tranfructosylation from sucrose, or hydrolysis of chicory inulin
Galacto-oligosaccharides	Oligo-galactose (85%), with some glucose and lactose	Produced from lactose by β-galactosidase
Soya-oligosaccharides	Mixture of raffinose (F-Gal-G) and stachyose (F-Gal-Gal-G)	Extracted from soya bean whey
Xylo-oligosaccharides	β-(1-4)-linked xylose	Enzymic hydrolysis of xylan
Pyrodextrins	Mixture of glucose-containing oligosaccharides	Pyrolysis of potato or maize starch
Isomalto-oligosaccharides	α-(1-4) glucose and branched α-(1-6) glucose	Transgalactosylation of maltose

DP, degree of polymerization; F, fructose; Gal, galactose; G, glucose

Adapted from Macfarlane, Macfarlane and Cummings (2006)

So far, the vast majority of studies on prebiotics have been focused on inulin, fructo-oligosaccharides (FOS) and galacto-oligosaccharides / transgalactosylatedoligosaccharides (GOS/TOS) (Macfarlane, Macfarlane and Cummings 2006). It has been proposed that the classification of two generations of prebiotics: the first generation of prebiotics possesses the bifidogenic effects and fibre-like properties on bowel inhabitant; the second generation of prebiotics focuses on specific biomarkers in lipid and mineral metabolism as well as immune enhancement and colon disease prevention (Ouwehand et al. 2005).

1.2.2 Beneficial health effects of prebiotics

Prebiotics are resistant to digestion in the host. Any carbohydrate reaching the large bowel will provide a substrate for the commensal microbiota through effects on their growth and metabolic activities (Macfarlane, Macfarlane and Cummings 2006). Although the health effects vary according to specific prebiotic and particular human object selected, there are published evidence for prebiotic effects on gut function and human health. Some prebiotics, such as galactooligosaccharide and inulin, could enhance the absorption of calcium so that lead to an increase in bone mineral density (Chonan and Watanuki 1996). Lactulose is known for its treatment of hepatic encephalopathy, as the nitrogenous compounds in the large bowel was uptaken by the bacteria through fermentation of lactulose and the fermentation products (Weber Jr. et al. 1987). Components of dietaey fibre, especially the highly fermentable ones, are trophic to the colonic epithelium and could enhance the mucosal proliferation in an

in vivo study (Jacobs and Lupton 1984). LDCs may function as protective factors, together with other metabolites from fermentation (particularly the butyrate), in combat with the detrimental factors in colorectal carcinogenesis (Hague et al. 1993). The remaining LDCs, which escaped fermentation by colonic microbiota, act by their water-holding capacity so as to exert a faecal bulking effect in the distal colon, and may help in the treatment of chronic functional constipation, symptomatic diverticulosis and the irritable bowel syndrome. The health related effects of LDCs in different region of human GIT are summarized in Figure 1.4 (Scheppach, Luehrs and Menzel 2001).

The future development of prebiotics should take a more integrative approach to modulate the microbial balance along the GIT and to produce direct health benefits on different regions of the human GIT.

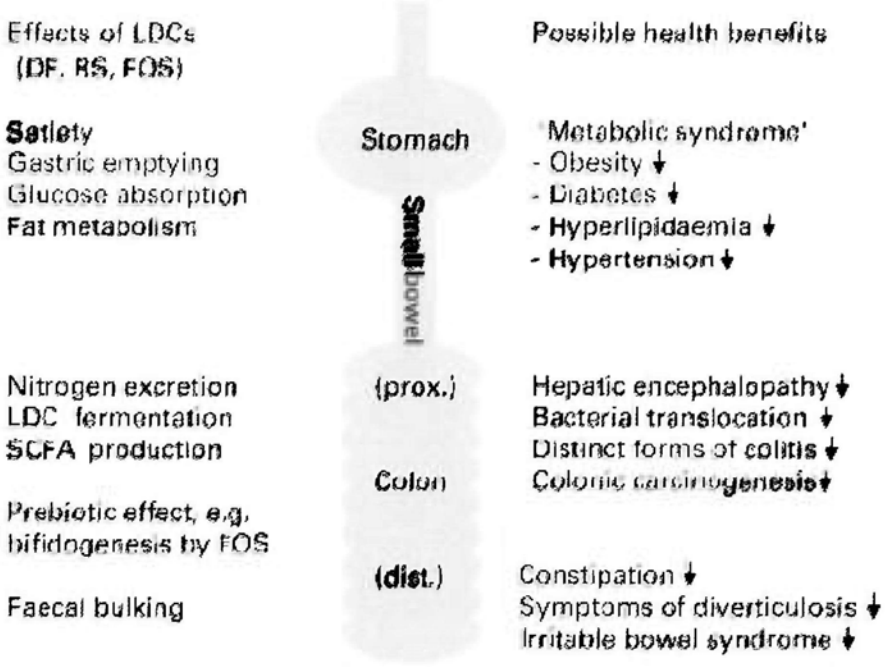

Fig. 1.4 Physiological effects of low-digestible carbohydrates (LDCs)
Left column: LDCs; Right column: LDC related health effects. DF: dietary fiber; RS: resistant starch; FOS: fructo-oligosaccharides; SCFA: short-chain fatty acids
Adapted from Scheppach, Luehrs and Menzel (2001)

1.3 Mushroom β-glucan

1.3.1 Beneficial effects of mushrooms

Mushroom has a long history of cultivation, which was started around 600 A.D. in China. The medicinal properties of mushrooms firstly recognized nearly 2000 years ago in China have been validated by studies all over the world (Kues and Liu 2000). Mushrooms received great interest since the late 1980s for they could produce a large number of biological active compounds, including polysaccharides, polyphenols, glycoproteins, triterpenes and antibiotics. Among these, polysaccharides are being the most extensively studied ones, with β-glucans being the most common subject (Aida et al. 2009).

Polysaccharides from mushrooms were suggested to have immunomodulating effects through enhancement of lymphocyte proliferation and antibody production (Bao et al. 2001). Polysaccharides are also known for their antitumor activity. It has been reported the β-glucans from mushroom sclerotia of *Pleurotus tuber-regium* could inhibit the proliferation of hepatic cancer cells (Tao, Zhang and Cheung 2006). It was found that a novel water-soluble polysaccharide from the fruiting body of *Pleurotus ostreatus* had showed antitumor activity against Hela tumor cells *in vitro* in a dose-dependent manner, and it exhibited significantly lower cytotoxicity to human kidney cells, making it a potential novel antitumor agent (Tong et al. 2009).

It has been proved that the backbone of the β-glucans from mushrooms have to be β-(1→3) linkages with β-(1→6) branches in order to have their antitumor

activities, although the activities also depend on many other factors including molecular weight, solubility in water, branching ratio and conformation (Aida et al. 2009). The mechanism of the antitumor activities may be various, including inhibition the proliferation of tumor cells, induction of apoptosis and cell cycle arrest, inhibition of invasive behavior and suppression of tumor angiogenesis, and etc. (Aida et al. 2009).

Besides antitumor activity, mushrooms have been documented for several other kinds of activities beneficial for health. It had been demonstrated that the aqueous extract from Shiitake (*Lentinula edodes*) mushrooms had antimicrobial activity against most of the microbial organisms tested including 29 bacteria and 10 fungi pathogens (Hearst et al. 2009). It was found that the extracts from three mushrooms including *Clitocybe maxima*, *Pleurotus ferulae* and *Pleurotus ostreatus* all exhibited antioxidant properties with phenolic compounds as the major active compounds (Tsai et al. 2009). Edible mushrooms are low in calories, sodium, fat and cholesterol but high in carbohydrate, protein, fiber, vitamins and minerals, indicating their high nutritional value as well as potential pharmaceutical value (Aida et al. 2009).

1.3.2 Mushrooms as potential source of prebiotics

In recent years, more and more attention has been paid to the physiological properties of food, and there is increasing demand for the development of prebiotics. As mentioned above, mushrooms own so many beneficial attributes to health and

contain high percentage of non-digestible carbohydrates, which classify mushrooms as a good potential prebiotic source. The carbohydrates from mushrooms may contain glucose, arabinose, mannose, fucose, galactose, xylose in different combinations in their chains along with various linkages. Table 1.8 summarized the sources, structures and reported bioactivities of some mushroom polysaccharides (Zhang et al. 2007). Although chitin, hemicellulose, mannans, xylans and galactans have been extracted from mushrooms, most of the mushroom carbohydrates belong to the group of β-glucans (Wasser 2002). Digestive enzymes secreted in human GIT are unable to hydrolyze β-glucosidic linkages. The resistance of mushroom β-glucans to digestion in the GIT by mammalian enzymes qualified them partially as prebiotic (Gibson 2004b). It was also reported that β-glucans from mushroom sclerotia (*Poria cocos* and *Polyporus rhinocerus*) stimulated the proliferation of *Lactobaccillus brevis* and *Bifidobacterium longum* while at the same time inhibited *Clostridium celatum* (Gao, Lai and Cheung 2009). This selectivity partially qualified β-glucans to be a potential prebiotic candidate. But further extensive studies need to be carried out before a full claim of mushroom β-glucans as a prebiotic can be made.

Table 1.8 Sources, structures and activities of some reported mushroom polysaccharides

Mushroom source	Polysaccharide source	Type	Main bioactivity
Pleurotus tuber-regium	Sclerotium, mycelium	β-D-glucan	Anti-breast cancer
Ganoderma lucidum	Fruiting body, culture broth	Heteroglycan, mannoglucan, glycopeptide	Hyperglycemia, antitumor, antioxidative
Schizophyllum commune	Mycelium	Glucan, schizophyllana	Antitumor
Hericum erinaceus	Fruiting body, mycelium	Heteroglycan, heteroglycanpeptide	Hyperglycemia, immunomodulating, antitumor
Lentinus edodes	Culture broth, fruiting body	Mannoglucan, polysaccharidee protein complex, glucan, lentinan	Immunomodulating, antitumor, antiviral

Adapted from Zhang et al. (2007)

1.4 Colonic fermentation and short chain fatty acid (SCFA)

1.4.1 Bacterial fermentation of NDCs in the colon

A large microbial population is present in the human colon at a level of 10^{10} to 10^{11} cfu/g wet weight with more than 50 genera and over 400 species of bacteria being identified in human faeces (Wong et al. 2006). The predominant species are non-spore forming anaerobes belonging to the genera *Bacteroides, Eubacterium* and *Bifidobacterium* (Cummings and Macfarlane 1991).

The anaerobic breakdown of carbohydrates and proteins by bacteria is defined as fermentation (Cummings and Macfarlane 1991). A variety of reactions and metabolic processes are involved in fermentation for the anaerobic breakdown of organic substrates, yielding metabolizable energy for microbial maintenance and growth together with other end products for use by the host (Wong et al. 2006). Figure 1.5 illustrates the main events during colonic fermentation and their metabolic process of the end products (Cummings and Macfarlane 1991b). Saccharolytic bacteria ferment the non-digestible carbohydrates (NDCs) in the colon and produce linear short chain fatty acids (SCFAs), hydrogen, carbon dioxide and this process could influence the colonic physiology in a beneficial way. Proteolytic bacteria degrade the protein and amino acids in the colon into branched SCFAs, methane, phenols and amines, which are harmful to the host (Macfarlane, Macfarlane and Cummings 2006).

Short chain fatty acids (SCFAs) are organic fatty acids containing 1 to 6 carbon

atoms and are the major anions produced from bacterial fermentation of polysaccharide, oligosaccharide, protein, peptide, and glycoprotein precursors in the colon (Wong et al. 2006). SCFAs have drawn more and more attention with the development of prebiotics and probiotics aiming at the improvement of colonic and holistic health.

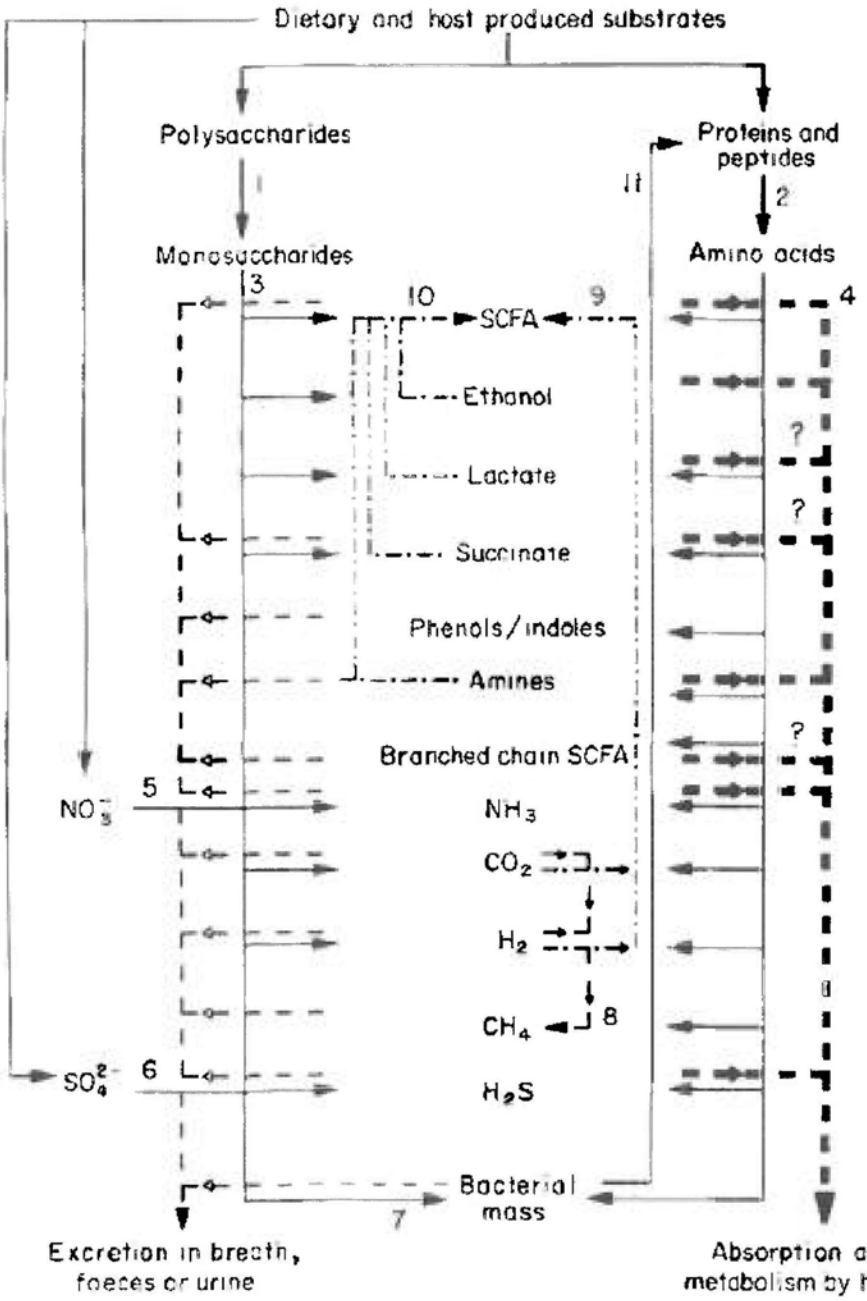

Fig.1.5 Summary of fermentation process and metabolic fate of the end products Adaped from Cummings and Macfarlane (1991b)

Various studies show that the SCFA production in the colon is in the order of acetate > propionate ≥ butyrate with a molar ratio of nearly 60 : 20 : 20, which remains fairly constant through different regions of the colon in spite of dietary changes (Wong et al. 2006).

Measurement of the fermentation end products showed that obvious regional differences in the fermentation activities occur in different regions of the colon (see Fig 1.6) (Cummings and Macfarlane 1991b). The production of SCFAs depend on a number of factors, including the numbers and types of microflora present in the colon, the types and structures of the substrates and gut transit time. Bacterial population, proliferation and fermentation are the highest in the proximal colon where substrate availability is the greatest, and fall progressively towards the distal colon. The colonic fermentation mainly occurs in the cecum and proximal colon, whereas the distal colon is short of carbohydrate and water. Accordingly, pH is at the lowest in the proximal colon and rises to about 6.9 (Cummings and Macfarlane 1991b).

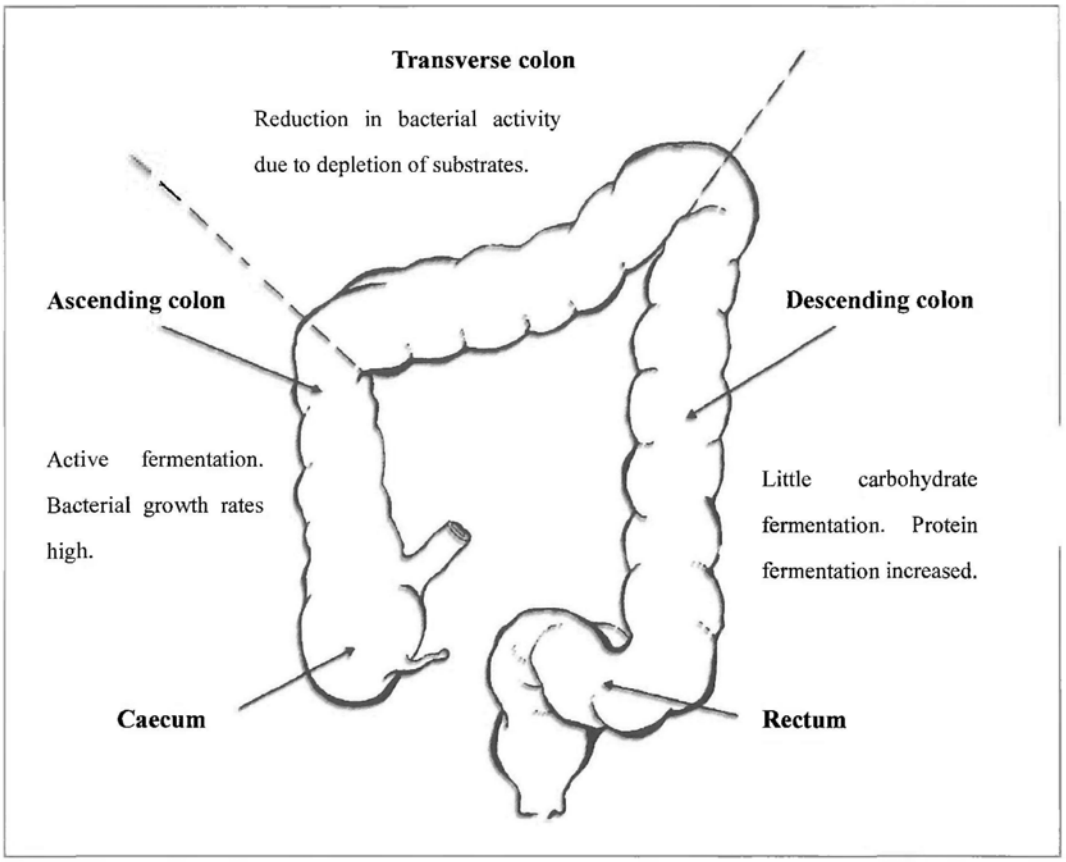

Fig. 1.6 Regional differences in fermentation activities of colonic microbiota Adapted from Cummings and Macfarlane (1991b)

Carbohydrates enter the colon mainly in the form of polysaccharides, which have not, or cannot be hydrolyzed by the digestive enzymes in the small intestine. The major group of polysaccharide-degrading bacteria may be Gram-negative *Bacteroides*, which are able to grow on a variety of polysaccharides, through synthesis of a wide range of cell-associated polysaccharide depolymerases and glycosidases (Macfarlane et al. 1990). Limited is known of the activities of other polysaccharide-degrading species in the colon, such as the bifidobacteria. It seems likely that the breakdown of highly polymerized carbohydrates in the colon is a cooperative activity, with enzymes from many different species participate (Cummings and Macfarlane 1991b).

Although many different carbohydrate sources are used as fermentation substrates in the colon, these substrates are degraded in a relatively small number of biochemical pathways, as shown in Fig 1.7 (Macfarlane and Macfarlane 2003). The majority of intestinal bacteria use the glycolytic pathway to derive energy from non-digestible carbohydrates (NDCs), while the fructose-6-phosphate pathway adopted by bifidobacteria has a restricted taxonomic distribution in the colonic microbiota (De Vries, Gerbrandy and Stouthamer 1967). The intermediate products: pyruvate and acetyl-CoA, are key control points in fermentative process, which can be converted into a wide range of end products (Macfarlane and Macfarlane 2003).

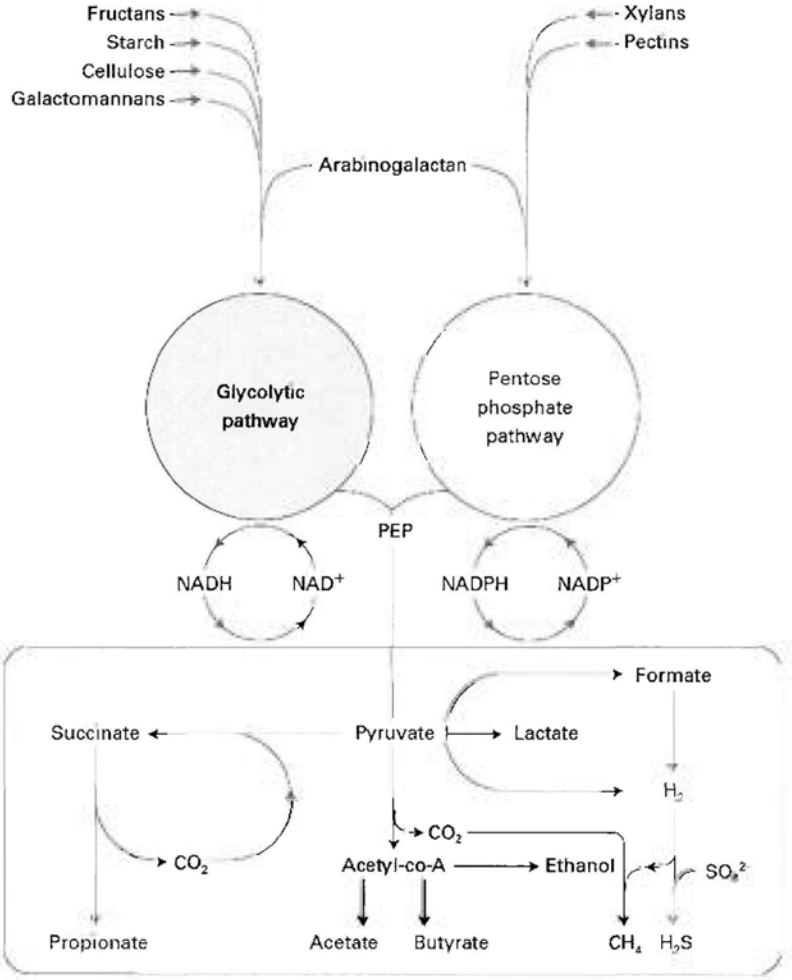

Fig. 1.7 Simplified representation of the major fermentation pathway of non-digestible polysaccharides in the colon. PEP: phosphoenolpyruvate
Adapted from Macfarlane and Macfarlane (2003)

1.4.2 Physiological importance of SCFAs

SCFAs produced from the fermentation in the colon are rapidly absorbed by the host with only 5 % to 10 % being excreted in the feces. Two proposed mechanisms may be involved: (1) diffusion of protonated SCFAs and (2) anion exchange (Cook and Sellin 1998). Once absorbed, SCFAs are metabolized mainly in 3 major regions of the host: (1) the colonic epithelium use butyrate as a major substrate for maintenance of energy production; (2) liver cells use residual butyrate with propionate for gluconeogenesis and take up more than half of the acetate; and (3) muscle cells could generate energy from the residual acetate (Wong et al. 2006). SCFAs exert their effects in many different ways including providing nutrients for the colonic epithelium; modulating pH in the colon and the composition of the colonic microbiota; increasing the absorption of minerals; regulating colonic cell proliferation, differentiation and gene expression; reducing the absorption of harmful compounds, etc. There are mounting studies on SCFAs regarding their key role in colonic health and their beneficial effects in certain diseases (Topping and Clifton 2001). Figure 1.8 is an illustration of the potential health benefits of SCFAs from colonic fermentation of prebiotics (Aachary and Prapulla 2011b). Together with probiotics and prebiotics, SCFAs display their potential influence on the balance of the host microflora, and in their enhancement on the function of the gut and immune system directly and indirectly. SCFAs from fermentation of prebiotics in the colon could enhance the mineral absorption, strengthen peristalsis, inhibit the growth of

pathogenic bacteria, reduce the harmful metabolites and reduce the risk of colon cancer, etc (Ruppin, Bar-Meir and Soergel 1980). Both *in vitro* and *in vivo* studies on the underlying mechanisms of these health benefits are needed (Wong et al. 2006; Aachary and Prapulla 2011b).

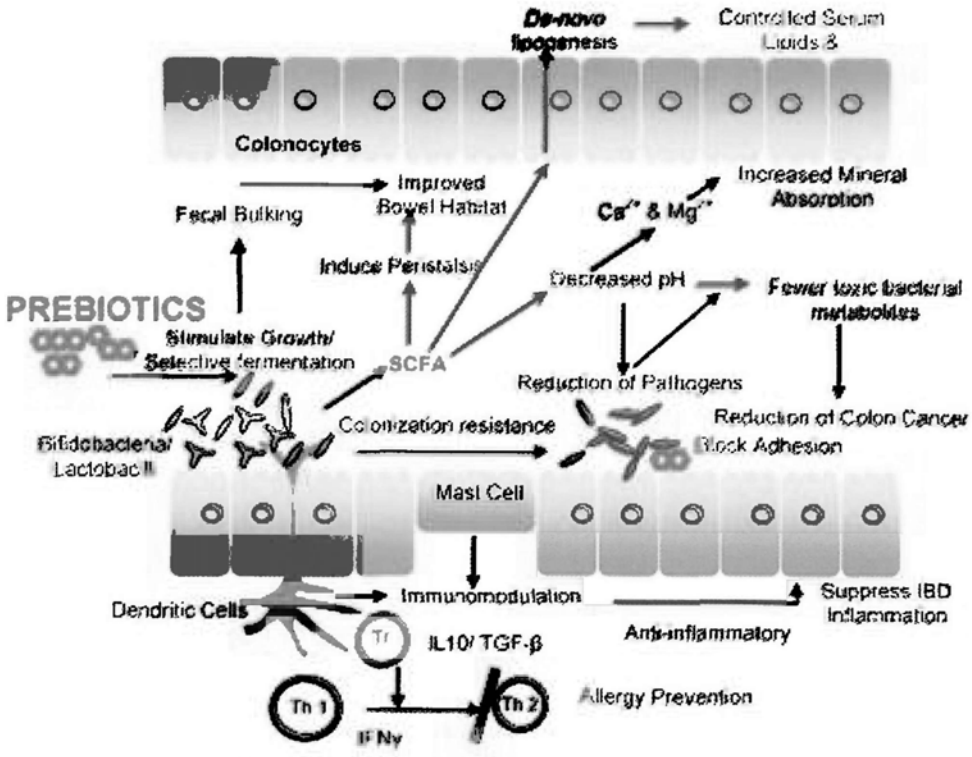

Fig. 1.8 Potential health benefits of SCFA from colonic fermentation
Adapted from Aachary and Prapulla (2011)

1.5 Previous studies on the bacterial fermentation of β-glucans from different sources

Until now, only a few studies have been carried out on the fermentability analysis of β-glucans originated from different sources by bacteria. It had been reported that laminarin isolated from *L. digitata* could be highly fermented by human fecal inoculum as most of the substrates disappeared after 24 h and a total SCFA concentration of 85 mM was produced with over 60 % of the SCFAs being acetic acid after 24 h (Michel et al. 1996). In another study, dietary curdlan from *Alcaligenes faecalis* was used to feed the rats and the cecal contents were analyzed after 4-week treatment (Shimizu et al. 2001). There had been significant increases in the bifidobacterial proliferation as well as the production of SCFAs and lactate in the cecum of curdlan-fed rats (Shimizu et al. 2001). Besides, the supernantant of the homogenized cecal content could enhance the growth of five species of bifidobacteria including *B. adolescentis*, *B. infantis* and *B. longum*, which indicated that bifidogenic factors might be released in the cecum of curdlan-fed rats (Shimizu et al. 2001).

Cereals is a common source of β-glucans, from which the β-glucans usually contain mixed β-1, 3 and β-1, 4 glycoside linkages in the sugar chain. Fermentation of β-glucans from barley and oats had been evaluated by the human faecal microbiota in terms of bacterial enumeration and production of SCFAs and lactic acid (Hughes et al. 2008). These β-glucans could significantly modulate the

microbial community but could not display significant bifidogenic effects. The high proportion of propionic acid in the SCFA profile of cereal β-glucans by human faecal fermentation might exert hypocholesterolaemic effects on the human GIT (Hughes et al. 2008).

In another study, β-glucans from three mushroom sclerotia were selected for the *in vitro* fermentation analysis by human fecal homogenate (Wong et al. 2005). All mushroom β-glucans were utilized in 24 h to different extent and the β-glucan from *Wolfiporia. cocos* gave the highest amount of SCFA at 5.23 mmol/g NDC. These results demonstrated the variations in the fermentability of mushroom β-glucans due to their possible structural arrangement and cell wall components (Wong et al. 2005). β-Glucans from *W. cocos* and *Polyporous rhinocerus* were also shown to stimulate the growth of *L. brevis* and *B. longum* but inhibited the proliferation of pathogenic bacteria *C. celatum* in a 24-h fermentation (Gao, Lai and Cheung 2009).

1.6 Objectives

β-Glucans from various sources have established beneficial effects on the human health as immunomodulating agents, but there have been very limited studies on their fermentability by human colonic bacteria. Moreover, a lack of the comparison of the fermentability among *β*-glucans made it difficult to evaluate the prebiotic potential of these *β*-glucans. This study compared the bifidogenic nature of the *β*-glucans obtained from different origins and explored the underlying mechanism that can explain the differences in fermentation by bifidobacteria.

The design and approaches of this project are as follows:

(a) *In vitro* fermentation study: the fermentability of *β*-glucans from seaweed, barley, bacteria and mushroom by Bifidobacterium pure cultures, including *B. adolescentis*, *B. infantis* and *B. longum*, was evaluated in a 24 h batch fermentation system with inulin as the control. The process was monitored in terms of pH, bacterial proliferation, organic matter disappearance and SCFA production. The fermentation pattern was compared to find out the difference between substrates.

(b) Comparative proteomic study: based on the fermentation results, bifidobacteria and *β*-glucans were selected for a comparative proteomic study. After 24 h incubation of bifidobacteria and *β*-glucans, the total proteins from model bifidobacterial cells were extracted and underwent two-dimensional difference gel electrophoresis (2D-DIGE) to reveal the changes in the protein expression profile in different fermentation systems. The differentially expressed proteins were selected, treated

and subjected to mass spectrometry (MS) analysis for identification. The annotations of the proteins would give a clue to their functional roles during fermentation.

(c) Real-time RT-PCR study: The total RNAs were extracted from the bifidobacterial cells incubated with different β-glucans for RT-PCR analysis. Based on the above information, the genes coding for those proteins expected to play an important role in carbon catabolism were selected for analysis at the transcription level.

(d) Enzyme activity study: The glucanase activity was assayed by the spectrophotometric method to test for the presence of extra- and intracellular bacterial enzyme activity in both the cell-free fermentation broth and the bacteria cells, respectively.

All the results from the above studies collectively would provide some evidence for proposing a model for the fermentation of β-glucans by bifidobacteria. It was anticipated that this model would deepen our current knowledge on the correlation of structural properties of β-glucans with their bifidogenic effect, and facilitate the understanding of the carbohydrate uptake mechanism in probiotic strains for the development of new and improved prebiotics.

Chapter 2 Materials and methods

2.1 Materials

2.1.1 Commercial Non-digestible carbohydrates (NDCs)

Three commercial β-glucans were obtained for this study: laminarin from *Laminaria digitata* (Sigma, St. Louis, USA), Barley β-glucan (Megazyme, Wicklow, Ireland), curdlan from *Alcaligenes faecalis* (Megazyme, Wicklow, Ireland). Inulin was obtained from Dahlia tubers (Sigma, St. Louis, USA).

2.1.2 Mushroom sclerotia and NDCs

2.1.2.1 Preparation of mushroom sclerotia

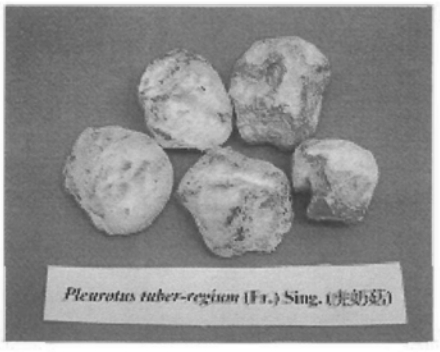

Fig. 2.1 The sclerotia of *Pleurotus tuber-regium*

Mushroom sclerotia from *Pleurotus tuber-regium* (PTR) were provided by the Sanming Mycological Institute in Fujian, China. The previously dried sclerotia were peeled and ground into powder by a mechanical grinder (MF10, IKA, USA) equipped with a 0.5 mm sieve.

2.1.2.2 Extraction of non-digestible carbohydrates (NDCs) from mushroom sclerotia

The extraction method was according to Gao et al (2009). Twenty grams of the mushroom sclerotium (PTR) powder from 2.1.2.1 were refluxed with 300 ml ethanol (80 %) for one hour to remove simple sugars and other low molecular weight substances. After centrifugation of 10000 *g* for 30 min, the pellets were soaked in NaOH (1 M) in a ratio 1:50 (v/v) for 48 h at ambient temperature. The mixture was then subjected to ultrasonication (Sonics VCX600) with an output of 40 % maximum amplitude for 40 min in an ice bath. The mixture was centrifuged at 10000 *g* for 30 min. The supernatant was dialyzed until most of the salt was removed to allow salt removal and finally lyophilized to give sclerotial NDCs of *P. tuber-regium* .

2.1.3 Human colonic bacteria

Pure cultures of Bifidobacteria used in this study including *Bifidobacterium longum* (JCM 1217), *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) (JCM 1222), and *Bifidobacterium adolescentis* (JCM 1275), were from Japan collection of Microorganism (JCM) and stored at -80℃. The three bifidobacteria were activated in Reinforced Clostridial Broth (RCB) (Oxoid, Hampshire, UK). 'Medium for colonic bacteria' (MCB) was used for the subsequent *in vitro* fermentation with or without NDC substrates supplemented.

2.2 Characterization of NDCs (β-glucans) from different sources

2.2.1 Determination of protein content by Kjeldahl method

The protein content of the NDCs extracted from mushroom sclerotia was determined by the Kjeldahl method. Half a gram samples and one piece of Kjeltab (potassium sulfate-copper sulfate) were placed in a Kjeldahl flask and 12 ml sulfuric acid (18 M) was added slowly with gentle shaking. The mixture was heated in Kjeldahl digestion apparatus at 420 ℃ until the solution became clear and continued for another 30 minutes. When the oxidation was completed, the flask was cooled in the fumehood to room temperature. After added with 75 ml distilled water, the flask was put in the Kjeltec system 1002 distilling unit. fifty ml 40 % sodium hydroxide was added slowly into the flask to make the solution alkaline. Ammonia was released from the mixture through bubbling by steam. The ammonia was captured by boric acid solution in a conical flask with its pH adjusted to the methyl red end point. Finally, the alkaline in the boric acid was titrated with hydrochloric acid (0.05 N).

The nitrogen percentage in the original sample was calculated as follows:

% Nitrogen = 〔vol. of acid (ml) × normality of acid × 1.4〕 / weight of sample (g)

Protein content = % nitrogen × 6.25

6.25 is the conversion factor for converting N to crude protein.

2.2.2 Determination of β-glucan content

The concentration of β-glucans in the NDCs including those from mushroom sclerotia were determined by "Mushroom and Yeast β-glucan assay kit" (Megazyme International Ltd., Wicklow, Ireland). The assay was carried out following the assay procedure provided with the kit. In brief, sample was weighed to about 100mg and put into a capped culture tube. Concentrated hydrochloric acid (37 % v/v) (1.5 ml) was added to each tube and the mixture was stirred vigorously. The tubes were put into a water bath at 30 ℃ for 45 min. Distilled water (10 ml) was added to each tube and the mixture was stirred. With their caps loosened the tubes were put into a boiling water bath. After 5 min, the caps were tightened and the incubation continued for 2 h. The tubes were cooled to room temperature and 10 ml of 2 N KOH was added to each tube. The contents in each tube were transferred to a 100 ml volumetric flask using 200 mM sodium acetate buffer (pH 5.0) to wash the tube and adjust the volume. An aliquot of each suspension was centrifuged at 1,500 *g* for 10 min and 0.1 ml. The supernatant (in duplicate) were transferred to glass test tubes. A mixture of exo-1, 3-β-glucanase (20 U/ml) plus β-glucosidase (4 U/ml) in 200 mM sodium acetate buffer (pH 5.0) at 0.1 mL was added to the bottom of each tube and the mixture was incubated at 40 ℃ for 60 min. Glucose oxidase/peroxidase mixture (GOPOD) (3.0 ml) was added to each tube and the mixture was incubated at 40 ℃ for 20 min. Absorbance of all solutions was measured at 510 nm against a reagent blank.

2.2.3 Determination of the molecular weight (MW) of β-glucans by size exclusion chromatography (SEC)

A SEC system consisting of HPLC pumps (Waters, Milford, USA) and various size exclusion columns including a TSK gel G3000 PW column (30 cm x 7.5 mm i.d., Cat. # 8-05762, Supelco) with a PWH guard column (7.5 cm x 7.5 mm i.d., Cat. # 8-06762, Supelco); a TSK gel G5000 PW column (30 cm x 7.5 mm i.d., Cat. # 8-05764, Supelco) and a TSK gel G6000 PW column (30 cm x 7.5 mm i.d., Cat. # 8-05765, Supelco) was used to determine the MW profile of mushroom sclerotial NDCs. The MW profile given by a range of pullulan standards (MW from 5.9 kDa to 404 kDa) was used as calibration. The eluent used was 0.2M sodium chloride solution and the flow rate was set at 0.7 ml/min. Temperature of the column was controlled externally at 30℃ and each sample or standard was allowed to run for 30 min. Signals from each sample or standard were detected by a refractive index (RI) detector.

2.2.4 Determination of monosaccharide profile by Gas Chromatography (GC)

2.2.4.1 Sample preparation for GC

For acid depolymerization, about 15 mg of sample was hydrolyzed with 0.7 ml of 12 M concentrated sulfuric acid at 35℃ for 60 minutes with continuous stirring. The mixture was diluted to 2 M sulfuric acid with addition of 3.5 ml distilled water

followed by incubation in a boiling water bath for 60 min, after which, the hydrolyzate was cooled to room temperature.

For neutral sugar derivatization, 3 ml of the hydrolyzate was transferred to a new test tube with internal standard allose (1ml, 1 mg/ml; Cat. # 05750, Fluka). With the addition of 12 M ammonia, the hydrolyzate was made alkaline. Five microliters of octan-1-ol (Cat. # O-4500, Sigma) together with freshly prepared sodium boron hydride (0.2 ml, 200 mg/ml in 2 M ammonia; Cat. # S-9125, Sigma) were added to the hydrolyzate. The mixture was incubated at 40℃ for 30 min followed by addition of 0.4 ml of glacial acetic acid and vortex-mixed. An aliquot of the mixture of 0.2 ml was transferred to a new test tube. Both 0.3 ml of 1-methylimidazole (Cat. # 336092, Sigma) and 2.0 ml of acetic anhydride was added to acetylate the mixture at room temperature for 10 min. Five milliliters of distilled water was then added and cooled under tap water to room temperature. One milliliter of dichloromethane was added and stood for 10 min for phase separation. After the removal of the upper phase, the lower phase was washed twice with 1 ml of water. The lower phase was dried with anhydrous sodium sulfate and then stored in a vial at -20 ℃ before GC analysis.

2.2.4.2 Gas chromatography (GC)

The alditol acetates derivatives of the neutral sugars prepared above were quantified by gas chromatography (Hewlett-Packard 6890, USA) with an Alltech DB-225 capillary column (15 m x 0.25 mm i.d., 0.25 μm film) and oven temperature

program of initial temperature 170℃, followed by a temperature rise rate of 2℃/min to 220℃ with a final hold of 10 min. The temperatures of the injector and the detector were set at 270℃. The carrier gas was helium and detection mode was flame ionization detection. Internal standard was corrected for losses during the treatment. Monosaccharide calibrations include fucose, rhamnose, ribose, arabinose, xylose, mannose, galactose, glucose, glucosamine and galactosamine. The values for monosaccharides were expressed as polysaccharide residues (anhydro-sugars) by multiplying the amounts of pentoses with the factor of 0.88, hexoses with the factor of 0.90 and deoxypentoses with the factor of 0.89.

The amount of the monosaccharides was calculated as follows:

$$\text{Amount of individual monosaccharide (mg)} = \frac{\text{Peak area (monosaccharide) in sample x Amount of allose (mg)}}{\text{Peak area (allose) in sample}}$$

The corresponding percentages of different monosaccharides by weight in the samples were calculated as follows.

$$\text{Monosaccharide \% by weight} = \frac{(R_f \text{ x coefficient x dilution factor}) \text{ x Amount of monosaccharide (mg) x } 100\%}{\text{Sample weight (mg)}}$$

R_f – response factor determined from monosaccharide standard

The relative amount (normalized) of monosaccharide as % of total monosaccharide in the sample was calculated as follows:

$$\text{Normalized monosaccharide \%} = \frac{\text{\% of individual monosaccharide x } 100\%}{\text{Total \% monosaccharide in the sample}}$$

2.2.5 Linkage analysis by gas chromatography mass spectrometry (GC-MS)

The sugar linkage of the β-glucans was studied by methylation analysis (Blakeney et al. 1983; Anumula and Taylor 1992) according to previous protocols.

2.2.5.1 Preparation of methylsulfinyl methyl sodium ($CH_3SOCH_2^-Na^+$)

Dry DMSO (≧99.9 %, for molecular biology, Sigma) was prepared by stirring the DMSO together with molecular sieve (Acros, 4Å, pre-dried at 550 ℃ for 3 hours) at 4 ℃ in a 500 ml brown round bottomed bottle flushed with nitrogen.

A suspension of NaH in oil was weighed into a test tube and washed with 30 ml hexane twice, and the NaH was dried under argon after hexane was removed before use. Two grams of NaH powder and 30 ml dry DMSO were added into a new test tube. The color of the mixture would change to grayish green after leaving it for 30 min at room temperature. Otherwise, the mixture was sonicated at 25 ℃ for 1 hour until the mixture turned into grayish green color. The mixture was then transferred to a 5 ml serum bottle which was flushed with nitrogen, capped and stored at -20 ℃ before use.

2.2.5.2 Methylation

The NDC samples (about 4 mg) dried over phosphorus pentoxide overnight under vacuum was dissolved in 1 ml dry DMSO by vortex-mixing and sonicated at

room temperature for 30-50 min. If the NDC samples remained insoluble in DMSO after this treatment, premethylation was done by addition of 20 μL $CH_3SOCH_2^-Na^+$ to the mixture which was kept in an ice-bath until frozen, followed by the addition of 5 μl pre-cooled methyl iodide (MeI) (Merck). The solution was then sonicated for 60 min at room temperature after the addition of 0.4 ml $CH_3SOCH_2^-Na^+$ followed by adding 0.3 ml pre-cooled MeI with sonication for another 15-20 min at room temperature. The remaining solution was kept at room temperature overnight.

2.2.5.3 Extraction of methylated polysaccharide

The methylated polysaccharide in the solution from the above was neutralized by 0.3 ml distilled water for the excess $CH_3SOCH_2^-Na^+$ and extracted by adding 4 ml chloroform ($CHCl_3$, Lab-Scan). The chloroform layer was washed 3 times with 3 ml distilled water each time which was removed by centrifuging at 3000 *g* for 2 min. The organic solvent was evaporated under a stream of nitrogen at 40 ℃ after the removal of the remaining water by adding 3 ml 2,2-dimethoxypropane and 40 μl glacial acetic acid.

2.2.5.4 Acid depolymerization and preparation of aditol acetate derivatives

The residue from above was depolymerized by heating with 0.6 ml 2 M

trifluoroacetic acid (TFA) at 121 ℃ for 1 hour, after which the acid could be removed by flushing with a stream of nitrogen at 40 ℃. The residue was made alkaline by the addition of 0.5 ml 12 M ammonia solution. The partially methylated monosaccharide in the solution was reduced and acetylated according to the method described in 2.2.4 with 0.2 ml allose (5 mg/ml of 50 % saturated benzoic acid) as the internal standard.

2.2.5.5 Determination of partially methylated alditol acetates (PMAAs) by gas chromatography mass spectrometry (GC-MS)

The alditol acetates derivatives of the partially methylated monosaccharides were analyzed by a gas chromatography- mass spectrometry system (Agilent Technology, 5973 N) including a DB225 capillary column (Altech, 15 m * 0.25 mm) with an autosampler in electron impact (EI) mode. The oven temperature program of the GC was set with an initial temperature of 160 ℃ followed by a temperature rise of 4 ℃/min to 280 ℃ with an interface temperature of 250 ℃ and an injector temperature of 280 ℃. The carrier gas was helium with a flow rate of 1.0 ml/min and detection made by electron impact ionization in MS. The ion source temperature of the MS was set at 250 ℃. The ionization energy and the detector voltage was set at 70 eV and 1.5 kV, respectively with a mass range from 40 to 350. The mass spectrum of the resulting peaks was identified by referring to the NIST/EPA/NIT data base in the computer to identify and quantify individual PMAAs.

2.3 *In vitro* batch fermentation of NDCs

2.3.1 Fermentation conditions

In vitro fermentation of the different NDCs derived from different origins was conducted by a batch fermentation system under strict anaerobic conditions with *Bifidobacterium infantis* (JCM 1222); *Bifidobacterium longum* (JCM 1217); *Bifidobacterium adolescentis* (JCM 1275) from the Japan Collection of Microorganism. The bacterium strain was activated by 2 % Reinforced Clostridial Broth (RCB, Oxoid, Hampshire, UK). Fermentation was conducted in the medium for colonic bacteria MCB (Medium for colonic bacteria) (Van Der Meulen et al. 2006) which contained (per liter):

6.5 g bacteriological peptone, 5.0 g soy peptone, 2.5 g tryptone, 3.0 g yeast extract, 2.0 g KCl, 0.2 g $NaHCO_3$, 4.5 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.45 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MnSO_4 \cdot H_2O$, 0.005 g $FeSO_4 \cdot 7H_2O$, 0.005 g $ZnSO_4 \cdot 7H_2O$, 0.4 g cysteine-HCl, 0.005 g hemin, and 0.005 g menadione, 0.5 ml H_3PO_4 and 2 ml Tween 80.

Different NDCs were used as the sole carbon source at a concentration of 0.5 % (w/v) in the MCB medium. The pH of the medium was adjusted to 6.50 before autoclaving (210 kPa, 121 °C, 20 min). The activated bacteria were inoculated at a concentration of 0.8 % (v/v) in the autoclaved MCB and oxyrase was added at a concentration of 0.8 % (v/v) to maintain the anaerobic environment. The mixture of culture medium and bacteria were put in capped bottles on a gentle shaker at 37 °C and the headspace was flushed with argon for 1 min.

2.3.2 Monitoring of pH during fermentation

The accumulation of fermentation metabolites of bifidobacteria would lead to a drop in pH of the fermentation broth, so the monitoring of the pH value to some extent would indicate the fermentation process. In this study, pH was measured by a pH meter (Φ240 pH/Temp Meter, Beckman, USA) at 0 h and 24 h of fermentation.

2.3.3 Monitoring of bacterial growth

After 24-h fermentation, an aliquot of 200 μl of the fermentation broth was transferred into tubes for bacteria proliferation analysis by viable count. The samples were diluted serially with sterile 0.9 % NaCl solution, and poured plating onto Plate Count Agar (BD Difco). Agar plates were placed in anaerobic jars with AnaeroGen Oxyrase (Oxoid) inside and incubated at 37 °C for 48 h before colony counting. Another aliquot of 600 μl was collected and stored at -20 ℃ for SCFA analysis.

2.3.4 Short chain fatty acid analysis

The short chain fatty acids (SCFAs) (acetate, butyrate, propionate) were analyzed by gas chromatography as described previously with slight modifications (Wong et al. 2005). An aliquot of 600 μl the fermentation broth was centrifuged (4800 *g*) at 4 °C for 30 min, then an aliquot of 350 μl of the upper supernatant was transferred into a new eppendorf, followed by adding 82.5 μl meta-phosphoric acid (25 %, w/v) to acidify the SCFAs and 67.5 μl methyl pentanoic acid (4.0 mg/ml) as internal standard (catalog no. 6220601, Alltech, USA). The SCFAs in the mixture

were extracted twice with 500 µl of diethyl ether and the upper layer of the organic fraction were pooled and dehydrated with anhydrous sodium sulfate before being filtered through a 0.45 µm membrane into GC vials.

The SCFA content were quantified by a HP 6890 GC system equipped with a Quadrex 007-FFAP capillary column (30 m × 0.25 mm; i.d. 0.25 µm film). An oven temperature was programmed an initial temperature of 80 °C with a hold of 5 min, followed by a temperature rise of 5 °C/min to 180 °C with a final hold of 5 min at 200 °C. Two microliters of samples were injected with a split ratio of 14.3:1, and detected by flame ionization. A mixture of individual SCFA standards including ethanoic acid, propanoic acid, butanoic acid (SCFA standards kit, Alltech, PA), and 4 methyl-pentanoic acid was prepared in 25 % meta-phosphoric acid at a final concentration of 1.0 mg/ml for identification and quantitation. The amount of the SCFAs after 24 h fermentation was expressed as millimoles and individual SCFA was expressed on percentage basis.

The correction factors, CF_{SCFA}, for each SCFA was calculated according to the following equation:

$$\mathbf{CF_{SCFA} = (P_{IS} \times W_{SCFA}) / (P_{SCFA} \times W_{IS})}$$

Where P_{IS} = GC peak area for internal standard (4-methyl pentanoic acid);

W_{SCFA} = weight (mg) of individual SCFAs standard used in the assay;

P_{SCFA} = GC peak area for individual SCFAs standard;

W_{IS} = weight (mg) of the internal standard used in the assay.

The content (mmol/g of substrate on organic matter basis) of individual SCFA

produced by the substrates was calculated as follows:

Individual SCFA (mmol/g) = (CF_{SCFA} × P_{SCFA} × W_{IS} × S_{TOTAL} × FR_{TOTAL})/ (P_{IS} × S_{ASSAY} × FR_{ASSAY} × MW × W_S)

Where CF_{SCFA} is correction factor for individual SCFA;

P_{SCFA} is the GC peak area for individual SCFA in sample solution;

W_{IS} is the weight (mg) of internal standard in sample solution;

S_{TOTAL} is the total volume of supernatant from 0.6 ml of fermented residue and ranged from 0.6 ml to 0.4 ml in different samples because of different amounts of debris after centrifuged;

FR_{TOTAL} is the total volume of fermented residue and is equal to 20 ml;

P_{IS} is the GC peak area for internal standard in sample solution;

S_{ASSAY} is the aliquot of supernatant for assay and is equal to 0.35 ml;

FR_{ASSAY} is the aliquot of fermented residue for assay and is equal to 0.6 ml;

MW is the molecular weight (g/mol) of individual SCFA (60.05 for ethanoic acid, 74.08 for propanoic acid, 88.11 for butanoic acid);

W_S is the weight (organic matter, mg) of original sample.

2.3.5 Organic matter disappearance (OMD)

Organic matter disappearance (OMD) was analyzed to evaluate the utilization efficiency of the NDCs in each system. Firstly, the fermentation residues were dried in a 120 ℃ vacuum oven and weighed. After that they were burned to ash in a 600 ^{o}C furnace (Cole-Parmer, Illinois, USA) overnight. The OMD percentage could be

calculated according to the following equation (Gao, Lai and Cheung 2009):

$$\mathbf{OMD\ (\%) = \{[OM_{original}-(OM_S-OM_B)]/OM_{original}\} \times 100\ \%}$$

In which OM original is the mean weight of organic matter (difference between weight of dry matter and weight of ash) of NDCs before fermentation;

OMS is the average weight of organic matter (difference between weight of dry matter and weight of ash) of the non-fermented residue with NDCs added after fermentation;

OM_B is the average weight of organic matter (difference between weight of dry matter and weight of ash) in the blank control after fermentation.

2.4 Proteomic study

2.4.1 Extraction of proteins from bifidobacteria

The bifidobacterial cells from fermentation broth were collected and washed three times in low salt buffer (3 mM KCl, 1.5 mM KH_2PO_4, 68 mM NaCl, and 9 mM NaH_2PO_4) (Yuan et al. 2006). The cells were resuspended in lysis buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 2 %) (GE Healthcare). The cells underwent ultrasonication in lysis buffer for 10 min. The supernatant was collected by centrifugation at 12000 *g* for 10 min. TCA/acetone at 10 % (V/V) was added to the supernatant, and the mixture was stored at -20 ℃ overnight for protein precipitation. The suspension was centrifuged at 10000 *g* at 4 ℃ for 10 min and the supernatant was discarded. The pellet was washed with ice-cool acetone and centrifuged at 3000 *g* for 1 min in order to remove any remaining solution. The pellet was air-dried for 5 minute and resuspended in rehydration stock solution (8 M urea, 2 % (w/v) CHAPS, 0.002 % (w/v) bromophenol blue). Then, the solution was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 12000 *g* for 5 minutes to remove any insoluble components. Afterwards, the supernatant was collected and transferred into a new 1.5 ml microcentrifuge tube and stored at -80 ℃ before use.

2.4.2 Protein quantitation

Each protein concentration in the solution from the previous step was measured using PlusOne 2-D Quant Kit (GE Healthcare). A standard curve was prepared for

every quantitation using the 2 mg/ml bovine serum albumin (BSA) standard solution provided in the kit. The assay range is 0.5 – 50 μg. Half a milliliter of precipitant was added in each tube, including the standard curve tubes. The tubes were mixed briefly and then incubated at room temperature for 2-3 min. Then 500 μl co-precipitant was added to each tube, mixed by inversion and the tubes were centrifuged at least 10000 *g* for 5 min. A small pellet of protein would then be visible. In order to avoid resuspension, the supernatant was decanted immediately. The tubes were repositioned in the centrifuge with the cap-hinge and the pellet pointing outward. The tubes were then spinned down in order to bring the remaining fluid to the bottom of the tubes. The remaining supernatant should be completely removed. Then, 100 μl copper solution and 400 μl distilled water were added to each tube and the precipitated protein should be totally dissolved. One milliliter of working color reagent, which was prepared by mixing 100 parts of color reagent A with 1 part of color reagent B, was added to each tube. The tubes were mixed by inversion or vortex and incubated at room temperature for 15 – 20 min. Finally, the absorbance at 480 nm of all the tubes including the standard curve tubes was measured with distilled water as blank. A standard curve of the absorbance of the standards against the quantity of protein was plotted and the protein concentration of the samples could be deduced using the standard curve.

2.4.3 Two-Dimensional Gel electrophoresis

Isoelectric focusing (IEF) was performed on Ettan IPGphor III isoelectric

focusing system (GE Healthcare). The samples were quantified by PlusOne 2-D Quant Kit as mentioned in 2.4.2 were normalized to 300 μg. Rehydration stock solution and 0.5 %(v/v) Pharmalyte (GE Healthcare) of corresponding pH range were added to make up to a volume of 250 μl. The prepared samples were applied to the Immoboline DryStrip gels (13 cm, pH 3-10) by rehydration loading overnight.

Before the second dimension, the IPG strip was equilibrated at room temperature for 15 min in the SDS equilibration buffer containing 50 mM Tris-HCL pH 8.8, 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS and 0.002 % (w/v) bromophenol blue, with DTT added prior to use. The strip was then washed with Milli-Q water (Millipore) briefly and further equilibrated at room temperature for another 15 minutes in the SDS equilibration buffer containing 50 mM Tris-HCL pH 8.8, 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS 0.002 % (w/v) bromophenol blue and 2.5 % (w/v) iodoacetamide.

The strip was washed with Milli-Q water briefly again and put onto the 12 % separating gels of 1 mm thickness. The strip was coverd with 400 μl sealing solution with SDS electrophoresis buffer containing 25 mM Tris-base, 192 mM glycine, 0.1 %SDS, 0.5 % and 0.002 %(w/v) bromophenol blue. The agarose sealing solution was allowed to polymerize. The gels and the SDS electrophoresis buffer was put inside the electrophoresis systems (Bio-Rad) and run at 80 V for about 15 h.

After the second dimension run, the gel was washed with Milli-Q water and then stained with 100 ml Coomassie blue staining solution which contains 45 % methanol, 10 % acetic acid, 0.1 % Coomassie Brilliant Blue R350 for 1 h. The

gels were destained overnight using 50 ml destaining solution containing 45 % methanol, 10 % acetic acid in Milli-Q water.

2.4.4 Image analysis

After staining and destaining, the gels were scanned under visible light at 300dpi using an ImageScanner (Amersham Bioscience). Analysis was performed using the computer program, ImageMaster 2D Platinum 5.0 (Amersham Biosciences). Spots were detected automatically while manual spot editing and deleting was performed if necessary. Distinct spots were selected throughout the gel for alignment and matching of the spots after the mass-spectrometry analysis. Gels were studied in triplicate, and clear gel with distinct spots was used as the reference gel in difference comparisons.

2.4.5 In gel digestion

The Coomassie blue stained protein spots of interest were cut out. The spots were transferred to 1.5 ml microcentrifuge tubes, and destained with 50 mM ammonium bicarbonate, dehydrated with ACN and then dried completely using a SpeedVac (LABCONCO). Five µL of 50 mM ammonium bicarbonate containing 20 ng/µl 'Sequencing Grade modified trypsin (Promega)' was added to the protein spots for rehydration and protein digestion. The samples were incubated overnight at 37 ℃. Afterwards, 3 µl extraction buffer (75 % CAN, 2.5 % TFA) was added to the samples, followed by sonication for 10 minutes. 0.4 µl digested sample solution in

the microcentrifuge tube was then spoted onto mass-spec plate twice, and then 0.4 µl matrix (x-cyano-4-hydroxycinnamic acid) was spotted.

2.4.6 Mass spectrometric analysis by MALDI-TOF MS/MS

Mass spectrometric analysis of the protein solutions from gel spots was carried out on a MALDI-TOF/TOF tandem mass spectrometer ABI 4700 proteomics analyzer (Applied Biosystems, Foster City, USA). For acquisition of mass spectra, 0.5 µl samples were spotted onto a MALDI plate, followed by 0.5 µl matrix solution (4 mg/ml α-cyano-4- hydroxycinnamic acid in 35 % ACN and 1% TFA). Mass data acquisitions were piloted by 4000 Series Explorer™ Software v3.0 using batched-processing and automatic switching between MS and MS/MS modes. All MS survey scan were acquired over the mass range 800-3500 *m/z* in the reflectron positive-ion mode and accumulated from 2000 laser shots with acceleration of 20 kV. The MS spectra were internally calibrated using porcine trypsin autolytic products (*m/z* 842.509, *m/z* 1045.564, *m/z* 1940.935 and *m/z* 2211.104) resulted in mass errors of less than 30 ppm. The MS peaks (MH^+) were detected on minimum S/N ratio≥20 and cluster area S/N threshold ≥25 without smoothing and raw spectrum filtering. Peptide precursor ions corresponding to contaminants including keratins and the trypsin autolytic products were excluded in a mass tolerance of ± 0.2 dalton. The filtered precursor ions with a user-defined threshold (S/N ratio ≥50) were selected for the MS/MS scan. Fragmentation of precursor ions was performed using MS-MS 1kV positive mode with CID on and argon as the collision gas. MS/MS spectra were

accumulated from 3000 laser shots using default calibration with Glu-Fibrinopeptide B from 4700 Calibration Mixture (Applied Biosystems, USA). The MS/MS peaks were detected on minimum S/N ratio ≥3 and cluster area S/N threshold ≥15 with smoothing.

2.4.7 Database Search

The MS and MS/MS data were loaded into the GPS Explorer™ software v3.5 (Applied Biosystems, Foster City, USA) and searched against Swiss-Prot database (released on September 18, 2006) by Mascot search engine v1.9.05 (Matrix science, London, UK) using combined MS (peptide-mass-fingerprint approach) with MS/MS (DeNovo sequencing approach) analysis for protein identification. The following search parameters were used: monoisotopic peptide mass (MH^+); 800-3500 Dalton; one missed cleavage per peptide; enzyme, trypsin; taxonomy, Bacteria; p*I*, 0-14; precursor-ion mass tolerance, 50ppm; MS/MS fragment-ion mass tolerance, 0.1 Dalton; variable modifications, carbamidomethylation for cysteine and oxidation for methionine were allowed. Known contaminant ions corresponding to trypsin and keratins were excluded from the peak lists before database searching. The top ten hits for each protein search were reported.

2.5 Real time reverse transcription Polymerase Chain Reaction (Real time RT-PCR)

2.5.1 RNA isolation and quantification

Total RNA was extracted from bifidobacteria cells which were incubated in different fermentation systems for about 7 h using the kit 'SV Total RNA Isolation System' (Promega, USA), following the protocol provided with the product. Bifidobacteria cells were collected by centrifuge and washed twice with potassium buffer. The cells were frozen in liquid nitrogen and grounded using a mortar and pestle. After mixing with 175 μl RNA lysis buffer, 350 μl RNA Dilution Buffer was added, mixed by inversion and centrifuged at 13000 *g* in a microcentrifuge for 10 min. The clear lysate was mixed with 200 μl and transferred into the Spin Column Assembly followed by centrifuge (13,000 *g*, 1 min). The mixture remaining in the spin basket was washed by RNA washing solution. Freshly prepared DNase incubation mix was added onto the membrane in the basket and incubated for 15 min at 20 ℃~25 ℃. After that, 200 μl of DNase I stop solution was added to stop the DNA degradation reaction followed by centrifugation (13,000 *g*, 1 min). The mixture was washed with 600 μl and 250 μl RNA washing buffer separately. Nuclease free water (50 μl ~100 μl) was added into each membrane for each sample and centrifuged (13,000 *g*, 1 min) to collect the purified RNA.

The yield and purity of the isolated RNA were evaluated by spectrometric method and agarose gel electrophoresis. The RNA could be used only when the ratio

of A_{260}/A_{230} should be 1.8-2.2, and the ratio of A_{260}/A_{280} should be 1.7-2.1 measured by spectrophotometer, while at the same time, brilliant sharp bands could be observed on the ethidium bromide staining agarose gel. The collected purified total RNA was stored at − 70℃.

2.5.2 First strand cDNA synthesis

The purified RNA was transcribed into cDNA using the kit 'ImProm- II Reverse Transcription System' (Proomega, USA) following the protocol provided with the kit. In an Eppendorf tube, up to 1 μg of purified total RNA and the Random primer from the kit were combined in nuclease-free water to a final volume of 5 μl per RT reaction. The tubes were placed in a preheated 70 ℃ heating block for 5 min and immediately chilled on ice for at least 5 min. Each tube was spun down to maintain the original volume. In a sterile 1.5 ml microcentrifuge tube, the following components were mixed according to the protocol to allow 15 μl for each cDNA synthesis reaction: nuclease-free water, reaction buffer, $MgCl_2$, dNTP mix, Recombinant RNasin Ribonuclease Inhibitor and ImProm- II Reverse Transcriptase. This reaction mix (15 μl) was added into 5 μl RNA and primer mix for reaction in each tube. These tubes were placed into a controlled-temperature heating block equilibrated at 25 ℃ for 5 min. The tubes were then incubated at 42 ℃ for up to 1 h. To inactivate the reverse transcriptase, the tubes were incubated in a controlled-temperature heating block at 70 ℃ for 15 min. The reactions were maintained frozen at -20 ℃ for long-term storage.

2.5.3 Real-time RT-PCR

The PCR amplication was performed using the IQ^{TM} SYBR Green Supermix kit (Bio-Rad). The '2× SYBR Green Supermix' reagent contained 100 mM KCl, 40 mM pH 8.4 Tris-HCl, 0.4 mM of each dNTP, 50 U/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green Ⅰ, fluoresein and stabilizers (Bio-Rad). The reaction solution consisted of 2 μl cDNA templates, 2 μl oligonucleotide primers, 10 μl '2× SYBR Green Supermix reagent' and 6 μL sterile distilled water, to a final reaction volume of 20 μl. The oligonucleotide primers used in this assay are listed in Table 2.1 and the gene coding for the 16 S ribosomal DNA (rDNA) (Bli008) was used as internal control for the expression of which is relatively constant in bacteria (Yuan et al. 2006). RNA extraction from the different samples was carried out in triplicate, and each replicate was analyzed in three independent PCR runs. Real-time PCR was carried out on the Bio-Rad IQ5 Real Time PCR Instrument. The thermal cycling was programmed as: 95℃ for 3 min; 40 cycles of 95℃ for 10 s, 55 ℃ for 30 s, 72 ℃ for 7 s and a melting curve was immediately run at the end of this thermal cycling. The results were analyzed using the $2^{-\Delta\Delta CT}$ method of relative quantification to represent relative expression values (Gilad et al. 2010). The gene was regarded as regulated when the one-way analysis of variance (ANOVA) test gave a P value < 0.05.

The equations are described as follows:

$$L = 2^{-C_t}$$

$$\frac{2^{-C_t(GOI)}}{2^{-C_t(HKG)}} = 2^{-[C_t(GOI) - C_t(HKG)]} = 2^{-\Delta C_t}$$

$$\text{Fold}^{\text{GIO}} = \frac{\dfrac{2^{-\Delta C_t(GOI)}\,\text{expt}}{2^{-\Delta C_t(HKG)}\,\text{expt}}}{\dfrac{2^{-\Delta C_t(GOI)}\,\text{control}}{2^{-\Delta C_t(HKG)}\,\text{control}}} = \frac{2^{-[C_t(GOI) - C_t(HKG)]}\,\text{expt}}{2^{-[C_t(GOI) - C_t(HKG)]}\,\text{control}} = \frac{2^{-\Delta C_t}\,\text{expt}}{2^{-\Delta C_t}\,\text{control}} = 2^{-\Delta\Delta C_t}$$

L means the original expression level; **GIO** means Genes of Interest;

HKG : House Keeping Genes;

Expt : samples treated with different β-glucans;

Control : samples treated with inulin.

C_t: Cycle threshold

Table 2.1 Oligonucleotides used in this study for gene expression analysis (Real-Time RT-PCR)

Protein name	Nucleotide sequence
Bli 001 outer membrane porin 1a (Ia;b;F) [*Escherichia coli*]	AGTTTGTTGTCAGAATCG; TAGAAGGTATCGGTGATG
Bli002 ABC transporter related [*B.infantis*]	GATGTACTTCAAGATACGGGTGTA GTGAACCGTGCCAAGGAA
Phosphotransferase; Bli003 BAD_0166 PTS system, emzyme 1 [*B.infantis*]	CAATGGTGAGCAAGTCAT CATCCTCGATCTCAGCAG
Bli004 outer membrane protein F [*Escherichia coli* CFT073]	CCAGTTTGTTGTCAGAAT TAGAAGGTATCGGTGATG
Bli005 ATP binding protein of ABC transporter for sugars [*B. longum* NCC2705]	AACAAGCTCACCACGATT CGGGTCGATCTTGATCTT
Bli006 BL0597 glycogen phosphorylase; glycogen/starch/alpha-glucan phosphorylases [*B.infantis*]	AATCACCGCACCGAAGTC TTTGAGTGTATTTCAGTTGTTCAC
Bli008 *B. infantis* gene for 16S rRNA	GTAACGGTGGAATGTGTA TATCTAATCCTGTTCGCTC
Bli 010 Transketolase [*B. longum* NCC2705]	GGACCAGATGTACAGGTT TTCTTCATGTTCTCCGACTA

2.6 Enzyme activity assay during fermentation

2.6.1 Glucanase activity assay

The hydrolysis of different β-glucan substrates, including laminarin, barley β-glucan and the β-glucan *P. tuber-regium* was quantified according to the dinitrosalicycic acid (DNS) method (Miller 1959) by measuring the released reducing sugar by glucanase. This glucanase assay was carried out in a pH 8.0 phosphate buffer with the substrate concentration of 0.25 % in a reaction volume of 200 µl at 40 ℃ for 10 min. Under this condition, the amount of glucanase required for the production of 1 µmol reducing sugar per minute is defined as one unit of glucanase activity (Yang et al. 2008).

2.6.2 Glucosidase activity assay

The β-glucosidase activity assay was carried out with *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as substrate in 50 mM phosphate buffer at 40 ℃. After 10 min incubation, the reaction was stopped by adding saturated sodium tetraborate solution and the absorbance was read at 405 nm. One unit of β-glucosidase activity is defined as the the amount of glucosidase required for the production of 1 µmol of *p*NP per minute under this condition (Peralta et al. 1997; Yang et al. 2008).

2.7 Statistical Analyses

All statistical analyses were performed by using the software SPSS Statistics 17.0 (SPSS, Chicago, Illinois). All the data obtained were analyzed by one-way ANOVA and tests of significant differences were determined by using Tukey's multiple comparison at $P < 0.05$.

Chapter 3 Results and discussion

3.1 *In vitro* fermentation of NDCs (β-glucans) with bifidobacteria pure cultures

3.1.1 Chemical composition and structure of β-glucans

The content of β-glucan in the NDCs prepared from the mushroom sclerotium of *Pleurotus tuber-regium* was 80.8 % with some non-β-glucan carbohydrate (10 %) and small amounts of proteins (1 %) and ash (1 %) while that of curdlan, barley and laminarin all have more than 95 % β-glucan with small amount of protein ranged from 0.45 to 3 %.

β-Glucans from barley, bacteria and mushroom contained only glucose as their sugar components and only that from seaweed contained small amount of mannose (< 2 %) in addition to glucose (> 98 %). From the results of the glycosidic linkage analysis on the β-glucans by methylation study, it was found that β-glucans from barley were a linear chain polysaccharide with mixed 1→3 and 1→4 β-linkages in the ratio of 1:3 (Table 3.1). While β-glucans from both bacteria (curdlan) and seaweeds (laminarin) had a β-(1→3) linked linear chain, curdlan was unbranched and laminarin was highly branched (Table 3.1). The β-glucans from mushroom sclerotia had the most complicated glycosidic linkage composition when compared with the other β-glucans by having a highly branched main chain with mixed glycosidic 1→3, 1→4 and 1→6 β-linkages (Table 3.1).

The molecular weight (M_w) of the β-glucans determined by SEC also differed greatly, with that from barley having the largest M_w of 590 x 10^3 Daltons, followed by that of mushroom (96 x 10^3 Daltons) and seaweed (6 x 10^3 Daltons). The M_w of curdlan could not be determined in the present study due to its poor water solubility but its M_w had been reported to be about 10 to 30 x 10^3 Daltons (Chan, Chan and Tang 2006). The M_w of inulin was about 5 x 10^3 Dalton according to the specification provided by the supplier (Sigma).

Table 3.1 Normalized percentage of partially methylated alditol acetates of NDCs used in 24-h fermentation by bifidobacteria

Partially methylated sugar	Barley*	Bacteria	Seaweed	Mushroom	Linkage type
2,3,4,6-Me_4-Glc	3	1	25	49	→1) Glc
2,4,6-Me_3-Glc	22	99	69	14	→1) Glc (3→
2,3,6-Me_3-Glc	75	n.d.	n.d.	18	→1) Glc (4→
2,3-Me_2-Glc	n.d.	n.d.	n.d.	11	→1) Glc (4→, 6→
2,3,4-Me_3-Glc	n.d.	n.d.	2	5	→1) Glc (6→
2,4-Me_2-Glc	n.d.	n.d.	4	3	→1) Glc (3→,6 →

Me: methyl residues; Glc: glucose residue; n.d. not detected

* NDCs are represented by their source of origin

3.1.2 *In vitro* fermentation monitored by pH, bacteria proliferation and SCFA production

3.1.2.1 Changes in pH in the fermentation system.

In this study, the *in vitro* fermentation time was 24 h which should be sufficient to imitate the microbial degradation of NSPs *in vivo* (Wisker et al. 1998). All the *in vitro* fermentation systems showed a significant drop ($p < 0.05$) of at least 0.5 units in their pH values after 24 h which is most probably caused by the fermentation end products (SCFAs) (Table 3.2). For *B. infantis*, the largest drop in pH was found in inulin (decreased from 6.51 to 4.77), implying that inulin was most efficiently utilized by *B. infantis*. No significant difference in the pH value between different NDC substrates was found in the fermentation system containing *B. longum* and *B. adolescentis*. It had been reported that there was only a modest drop of pH value (0.1 to 0.3) during a 24 h fermentation of β-glucan rich oat samples with a β-glucan content of 5.2 to 7.7 % using human fecal flora (Kim and White 2009). The lowering of pH during fermentation could exert beneficial effects for colonic environment, such as inhibiting the growth of pathogenic bacteria and increasing the growth of beneficial ones (Cummings and Macfarlane 1991b; Wong et al. 2005).

Table 3.2 pH changes in batch cultures during 24 h *in vitro* fermentation of different NDCs by bifidobacteria

Bifidobacteria	Time	Dahlia tuber*	Barley	Bacteria	Seaweed	Mushroom
B. infantis	0	6.51±0.00	6.52±0.01	6.51±0.01	6.5±0.01	6.48±0.01
	24 h	4.77±0.01a	6.04±0.02cd	5.96±0.03b	6.02±0.00c	6.05±0.05cd
B. longum	0	6.24±0.02	6.28±0.01	6.30±0.01	6.28±0.01	6.33±0.01
	24 h	5.71±0.05a	5.81±0.01abc	5.83±0.01bc	5.78±0.05ab	5.85±0.03bc
B. adolescentis	0	6.50±0.06	6.49±0.01	6.51±0.01	6.52±0.00	6.52±0.01
	24 h	5.73±0.02bc	5.69±0.03ab	5.74±0.02bcd	5.78±0.01cd	5.76±0.01cd

Values are means ± standard deviations (N=3). Values in the same row followed by different letters are significantlydifferent (ANOVA Tukey's multiple comparison test $P < 0.05$)

* NDC is represented by the name of its source

3.1.2.2 Viability of bifidobacteria during fermentation of β-glucans.

Growth of bifidobacteria during the 24 h fermentation of β-glucans was monitored by viable count instead of by optical density (OD) measurement because the substrates in the fermentation broth caused interference to OD values. Among the 3 bifidobacteria, *B. infantis* increased in number by an average of 3 $\log_{10}$ CFU on all the NDC substrates with the highest increase of 4 $\log_{10}$ CFU in laminarin (Table 3.3). The increase in the populations of *B. longum* and *B. adolescentis* incubated with the different β-glucan substrates ranged from 1 to 1.5 $\log_{10}$ CFU and 2 to 2.3 $\log_{10}$ CFU, respectively (Table 3.3). The multiplication of the populations of the 3 bifidobacteria in inulin was found to be similar to that of all the β-glucan substrates (Table 3.3). In contrast to our observations, it had been previously reported that *B. infantis, B. aldolescentis* and *B. longum* did not grow in an *in vitro* fermentation system using barley β-glucan as carbon substrates (Crittenden et al. 2002).

While there were many factors causing such differences in the results, the use of different strains of bifidobacteria (strains were obtained from VTT Biotechnology (Finland) in the previous study (Crittenden et al. 2002) and were obtained from JCM (Japan) in the present study) might be one of the contributing factors. The present results were consistent with previous report that *B. adolescentis* and *B. longum* could grow in fructooligosaccharide (an inulin-type fructan) (Pastell et al. 2009). In summary, *B. infantis* had the highest population after 24-h fermentation in the β-glucan substrates, which might be partly explained by having the shortest

generation time (50 min) when compared to *B. adolescentis* (69 min) and *B. longum* (111 min) as reported previously (Lee, 2007).

Table 3.3 Bifidobacterial populations (log_{10} CFU per mL batch culture fluid) in batch culture during 24 h *in vitro* fermentation of different NDCs

Bifidobacteria	Hour	Dahlia tuber*	Barley	Bacteria	Seaweed	Mushroom
B. infantis	0	5.22±0.15	4.96±0.26	5.17±0.08	5.07±0.16	5.07±0.10
	24 h	8.60±0.05ab	8.63±0.20ab	8.77±0.19ab	9.25±0.01b	8.47±0.12a
B. longum	0	4.93±0.04	4.66±0.26	4.81±0.05	4.59±0.16	4.63±0.46
	24 h	6.33±0.17a	6.02±0.17a	5.95±0.07a	5.89±0.16a	6.02±0.17a
B. adolescentis	0	4.40±0.17	4.75±0.05	4.52±0.07	4.63±0.06	4.40±0.17
	24 h	6.77±0.13a	6.82±0.08a	6.84±0.11a	6.77±0.06a	6.66±0.03a

Values are mean values of (log_{10} viable count) ± standard deviations (N=3). Values in the same row with different letters are significantly different (ANOVA Tukey's multiple comparison test $P < 0.05$). * NDCs are represented by their source of origins

3.1.2.3 Organic matter disappearance after 24 h fermentation

Generally speaking, all the NDC (β-glucans) substrates were utilized by the bifidobacteria to some extent, ranging from 4.52 % to 41.3 % (Table 3.4). As the control, inulin was used extensively by *B. longum* with the highest ($p< 0.05$) OMD at 41.3 %. *B. infantis* and *B. adolescentis* also utilized the inulin to a large extent, at 23.0 % and 35.6 % respectively. Among the β-glucans, laminarin had an OMD of nearly 37 % when fermented by *B. infantis*, when compared to that of *B. longum* (23.0 %). All the OMD of *B. infantis* were over 10 % with the lowest value (13.5 %) being found in curdlan. Such high efficiency of utilization was consistent with the high growth rate of *B. infantis* during the 24-h fermentation period (Table 3.3). Except for laminarin and inulin, other β-glucan substrates were relatively less fermented by *B. longum*. For *B. adolescentis*, the OMD for laminarin and barley β-glucan were similar, both of which were over 10 %. However, *B. adolescentis* could hardly use curdlan and the β-glucans from mushrooms (PTR). Previous study had reported that the OMD for PTR was 9.82 % after 24h fermentation by human fecal homogenate (Wong et al. 2005). For *B. infantis*, OMD was negatively correlated with the results of pH (Table 3.2) and the microbial growth (Table 3.3), while no such correlation was found in the other 2 bifidobacteria.

Table 3.4 Organic matter disappearance in the different NDCs incubated with bifidobacteria after 24 h *in vitro* fermentation

OMD %	Dahlia tuber*	Barley	Bacteria	Seaweed	Mushroom
B.infantis	23.0±10.0	15.6±4.51	13.5±8.38	36.9±5.98	17.8±8.47
B.longum	41.3±2.38a	4.52±3.29c	5.27±7.55c	23.0±5.26b	5.51±2.30c
B.adolescentis	35.6±2.29a	13.0±1.82b	N	11.0±8.25b	N

Values are means ± standard deviation (N=3). Values in the same row followed by different letters are significantly different (ANOVA Tukey's multiple comparison test $P < 0.05$); N: no organic matter disappearance was observed. * NDCs are represented by their source of origin

3.1.2.4 Production of SCFAs during fermentation of β-Glucans

SCFAs are the major organic acids produced by colonic bacteria during the fermentation of polysaccharides, with acetic acid, propionic acid and butyric acid being the principal components (Cummings and Macfarlane 1991b). All three bifidobacteria produced various amounts of SCFAs as metabolites in the 24-h fermentation of the β-glucans in this study. The total SCFA production in *B. infantis* was higher than that in *B. longum* and *B. adolescentis* for all the β-glucans (Table 3.5). Compared with the β-glucans, *B. infantis* produced significantly ($p<0.05$) higher level of total SCFAs when incubated with inulin, which was also consistent with the largest drop in the pH value found (Table 3.2). The SCFA profile of *B. infantis* was very different from that of *B. longum* and *B. adolescentis* for all the β-glucans in that the ratio of acetic : propionic : butyric acid was 8:1:1. Acetic acid was predominantly produced in *B. longum* and *B. adolescentis*, accounting more than 96% of the total SCFAs while propionic acid and butyric acid only consitituted for about 2 and 1 %, respectively for all the β-glucans (Table 3.5). The SCFA profile for inulin was also dominated by acetate (96%) and was the same among all 3 bifidobacteria (Table 3.5).

Table 3.5 Production of total SCFAs (mM) and their normalized molar percentage (%) in batch culture fluid after 24 h *in vitro* fermentation of different NDCs by bifidobacteria

Bifidobacteria		Dahlia tuber*	Barley	Bacteria	Seaweed	Mushroom
B. infantis	**Total SCFA(mM)**	3.86±0.22a	1.97±0.33b	2.60±0.66b	2.00±0.03b	2.24±0.08b
	Acetic acid	95.06±0.72a	79.20±1.04b	80.48±0.39 b	81.14±1.77b	78.70±3.27b
	Propionic acid	2.82±0.27a	11.73±0.08b	11.21±0.71 b	10.91±0.79b	11.78±1.68b
	Butyric acid	2.12±0.44a	9.08±0.99b	8.32±0.33b	7.95±0.98b	9.52±1.59b
B. longum	**Total SCFA(mM)**	1.08±0.10	1.03±0.02	1.12±0.06	1.00±0.07	0.98±0.01
	Acetic acid	96.48±0.21a	96.32±0.18a	96.26±0.15a	96.29±0.27a	96.25±0.06a
	Propionic acid	2.22±0.12a	2.34±0.10a	2.38±0.24a	2.33±0.14a	2.30±0.01a
	Butyric acid	1.30±0.10a	1.34±0.07a	1.36±0.09a	1.38±0.13a	1.45±0.06a
B. adolescentis	**Total SCFA(mM)**	1.06±0.05a	1.18±0.10a	1.08±0.11a	1.08±0.04a	1.16±0.02a
	Acetic acid	96.38±0.05a	96.24±0.02a	96.14±0.38a	96.32±0.08a	96.40±0.16a
	Propionic acid	2.36±0.04a	2.43±0.01a	2.48±0.20a	2.35±0.06a	2.26±0.07a
	Butyric acid	1.26±0.09a	1.34±0.02a	1.38±0.22a	1.33±0.04a	1.34±0.09a

Values are means ± standard deviation (N=3). Values in the same row with different letters are significantly different (ANOVA Tukey's multiple comparison test $P < 0.05$). * NDCs are represented by their source of origins

3.1.3 Comparison of the fermentation of β-glucans by bifidobacteria

NDCs including non-starch polysaccharides and oligosaccharides are the most common prebiotics (Playne and Crittenden 1996). Sources of prebiotic NDCs are mainly from the cell wall of higher plants including cereals and grains, fruits and vegetables as well as legumes and seaweeds because they are resistant to hydrolysis by human alimentary enzymes (Velaquez et al. 2000). Moreover, the most extensively investigated prebiotic NDCs is inulin which is a naturally occurring glycan consisting of a linear chain of fructose residues with β-(2→1) glycosidic linkages (Gibson and Fuller 2000). Other emerging prebiotics include galacto-oligosaccharides (GOS) and xylo-oligosaccharides (XOS) which have received much attention recently (Torres et al. 2010) (Aachary and Prapulla 2011).

Prebiotic NDCs that are glucose-based are lesser-known with isomaltulose and β-glucans being the potential ones (Lina, Jonker and Kozianowski 2002). Some limited studies on β-glucans with mixed 1→3 and 1→4 β-glycosidic linkages from oat and wheat and gentio-oligosaccharides with β-(1→6) links have shown that glucose-based NDCs are also highly fermentable and can act as an alternative source of prebiotics (Rycroft et al. 2001; Wood et al. 2002).

Water-soluble β-glucans with different structural characteristics can be commonly found from different origins including algae (seaweed), cereal (barley), bacteria and fungus (mushrooms). Laminarin from *Laminaria digitata* is an algal (seaweed) storage polysaccharide having a linear β-(1→3)-linked glucan backbone

with β-(1→6)-linked branches (at the 3:1 ratio) (Read, Currie and Bacic 1996). Curdlan is an exo-polysaccharide produced by a non-pathogenic bacteria *Alcaligenes faecalis* and consists of a linear polymer with β-(1→3)-linked glucose residues that is used as gelling agent in food (Harada, Misaki and Saito 1968). The β-glucan from barley is a common cereal NDCs that consists of a mixed linkages of β-(1→3) and β-(1→4) in the main chain (Lambo, Oste and Nyman 2005). Fungal β-glucans found in mushrooms usually have a β-(1→3) linked glucose main chain with different branching ratios of side chains having β-(1→6) linked glucose (Wong et al. 2005).

All these different β-glucans have been shown to be bioactive (especially on immunomodulation) and have health benefits to humans (Novak and Vetvicka 2008). Recently, mushroom β-glucans isolated from sclerotia (multihyphal structures developed by the aggregation of specialized mycelia) have been shown to promote the growth of probiotics and are potential prebiotic candidates (Aida et al. 2009; Gao, Lai and Cheung 2009). The β-glucans from cereals (oat and barley) have also been recently evaluated for their effects to improve the viability of bifidobacteria strains in probiotic-containing yogurt (Vasiljevic, Kealy and Mishra 2007; Rosburg, Boylston and White 2010).

Laminarin is a linear polysaccharide with β-1, 6-branches for every three glucose units and is used as a common substrate for laminarinase (EC 3.2.1.6) activity assays. Complete hydrolysis of laminarin should involve β-1, 3 or β-1, 6 glucanase and β-glucosidase. It had been reported that laminarin isolated from *L. digitata* could be extensively fermented by human fecal inoculum to produce a total

SCFA concentration of 85 mM with over 60 % of the SCFAs being acetic acid after 24 h (Michel et al. 1996). In the present study, all three bifidobacteria exhibited high fermentability towards laminarin, implying that specific β glucanases could be induced in these bifidobacteria to hydrolyze the laminarin.

As mentioned earlier, curdlan is a linear β-1, 3-glucan with almost no branches and is very insoluble in water. Despite such low water solubility, curdlan produced by *A. faecalis* has been reported to markedly increase the *in vitro* proliferation of the same bifidobacteria used in the present study with *B. adolescentis* having the largest increase in population (Shimizu et al. 2001). While the three bifidobacteria could utilize curdlan to different extent, curdlan seemed to be more efficiently degraded by *B. infantis* as shown by the highest proliferation (Table 3.3) and largest SCFA production (Table 3.5).

Water-soluble β-glucans from cereals including oat and barley contain β-1, 3 and β-1, 4 mixed linkages that require specific β-glucanases (lichenase) with both β-1, 3 and β-1, 4 cleavage activity to degrade them completely (Hughes et al. 2008). The present results had demonstrated that barley β-glucans with a M_w of 590 x 10^3 daltons could be utilized by the 3 bifidobacteria with *B. infantis* having the highest proliferation (Table 3.3) and SCFA production (Table 3.5), suggesting that lichenase-type enzymes could be induced in the bifidobacteria. However, previous *in vitro* experiments using human faecal microbiota to ferment barley derived β-glucans with M_w between 137 to 327 x 10^3 daltons displayed no apparent prebiotic potential for these substrates (Hughes et al. 2008). More *in vivo* and *in vitro* investigations are

required to ascertain the bifidogenic properties of cereal β-glucans in relationship to its physico-chemical properties especially their molecular size.

The most common chemical structure of β-glucans from mushrooms is a β-1, 3 backbone with different degree of β-1, 6 and/or β-1, 4 branching (Zhang et al. 2007). The β-glucans isolated from the sclerotia of *P. tuber-regium* in the present study had a mixed linkages of β-1, 3, β-1, 4 and β-1,6 (Table 3.1) which was consistent with our previous study (Wong et al. 2005). In the present study, mushroom sclerotial β-glucan could be utilized similarly as the other β-glucans by pure cultures of bifidobacteria despite its complex linkages. It had been reported previously that β-glucans isolated from other mushroom sclerotia including *Polyporus rhinocerus* and *Poria cocos* could also stimulate the growth of *B. longum* and *Lactobacillus brevis* while inhibited the growth of *Clostridium celatum* (Gao, Lai and Cheung 2009). These results suggested that mushroom sclerotial β-glucans had the potential to be developed as a novel prebiotic.

In vivo studies had shown that inulin could be selectively and efficiently utilized by bifidobacteria including *B. adolescentis* and *B. longum* (Gibson et al. 1995) (Ramirez-Farias et al. 2009). The present results indicated that *B. infantis* could utilize inulin more efficiently than *B. longum* and *B. adolescentis* in pure cultures by having a lower pH value in the culture medium, a large increase in population and larger amount of total SCFAs (Table 3.2 to 3.5). These results were in agreement with the findings that bifidobacteria could degrade inulin and oligofructose by producing β-fructofuranosidase (Perrin et al. 2002).

The possible explanation for the present results can be inferred from the recent release of genome sequences of *B. infantis*, *B. longum* and *B. adolescentis* which would provide important information on the mechanisms of the fermentation of NSPs by these bifidobacteria in terms of their carbohydrate-modifying enzymes (carbohydrases) (NCBI). Those proteins (enzymes) identified from the bifidobacterium genomes which are responsible for the utilization of NSPs are summarized in Table 3.6. The three bifidobacterium species all possess several glucan hydrolases, mainly glucosidases. Besides, *B. infantis* possesses one glycogen phosphorylase, which belongs to a family of oligosaccharide phosphorylases that can convert the oligosaccharides from β-glucans into glucose-1-phosphate, facilitating the efficiency of their transfer into the central fermentative pathway (NCBI).

Another interesting phenomenon is that there are 21 family 1 solute binding proteins (SBPs) in *B. infantis* while there are only 10 and 11 of family 1 SBPs in *B. longum* and *B. adolescentis*, respectively (NCBI). Family 1 SBPs is a component of the ATP-binding cassette (ABC) transport system which is responsible for carbohydrate binding in the membrane (Sela et al. 2008). The relatively large number of SBPs found in *B. infantis* might in part explain its higher efficiency in the fermentation of the β-glucan substrates than the other two bifidobacteria. Moreover, in the case of *B. infantis*, the amount of total SCFA produced during the fermentation of inulin is almost twice the amount of that in the β-glucan substrates (Table 3.5) even though the bifidobacterial populations were similar (Table 3.3). This might be due to the fact that inulin has a relatively lower molecular weight and higher

proportion of oligosaccharides than that of the β-glucan substrates, allowing them to be more readily bound and transported by SBPs across the cell membrane for utilization.

Other mechanistic models have been proposed to explain the molecular mechanism of the utilization of NDCs by colonic bacteria (Sela et al. 2008). For example, *B. longum* possessed a multi-domain glycanase, which includes one transmembrane domain to anchor in the cell membrane and two carbohydrate binding modules as a catalytic domain to capture NDCs such as xylan oligosaccharide (Van Den Broek et al. 2008). In general, colonic bacteria such as bifidobacteria are capable of capturing and degrading NDCs in their surroundings and transport the polysaccharides or their hydrolytic products into the cells as energy sources (Van Den Broek et al. 2008).

In conclusion, all the three bifidobacteria used in the present study were capable of utilizing all the structurally-diversified β-glucans as substrate of fermentation that is comparable to the known prebiotic inulin. *B. infantis* seems to be able to grow better than the other two bidfidobacteria in all the β-glucans because of the larger number of relevant carbohydrases based on genome analysis of *Bifidobacterium* sp. in the literature. Further biochemical characterization to confirm their mode of action and substrate specificity are required. Experiments are required to demonstrate the molecular mechanisms by which bifidobacteria can specifically degrade β-glucans from different sources that have different chemical structures by measuring the corresponding enzyme activity in the fermentation broth. Further detailed

investigations (Section 3.2) were carried out to explain the apparent versatility of bifidobacteria to employ different strategies for utilizing different β-glucans.

Table 3.6 Summary of the number of proteins and their accession number identified in the bifidobacterium genome which may play a role in NDC degradation*

Proteins	*B. infantis*	*B. longum*	*B. adolescentis*
Beta-glucosidase	YP_002322110.1 YP_002323355.1	YP_004221256.1 YP_004221261.1	YP_910057.1 YP_910150.1 YP_910473.1 YP_910474.1
Glucan 1,3-beta-glucosidase	YP_002322106.1	nil	nil
Beta-1,3-exoglucanase	nil	YP_004221257.1	YP_910065.1 YP_910067.1
Glycogen/starch/alpha-glucan phosphorylase	YP_002321578.1	nil	nil
Solute binding protein family1	21	10	11

* Based on the information provided by NCBI database

B.infantis: *Bifidobacterium longum* subsp. *infantis ATCC 15697,* complete genome

B.longum: *Bifidobacterium longum* subsp. *longum JCM 1217,* complete genome

B.adolescentis: *Bifidobacterium adolescentis ATCC 15703* chromosome, complete genome

3.2 Mechanistic study of fermentation of β-glucans from different sources by *B. infantis*

3.2.1 Comparative proteome analysis of *B. infantis* incubated with different β-glucans through 2D-DIGE

B. infantis was selected as the model organism for the following mechanistic study because of the results in the batch fermentation (Section 3.1) in terms of SCFA production and individual SCFA ratios. *B. infantis* seemed to have specific metabolic pathways when incubated with β-glucans from different sources to give the differences observed in section 3.1 compared to those of *B. longum* and *B. adolescentis*.

The two-dimensional difference gel electrophoresis (2D-DIGE) gels containing the total proteins isolated from *B. infantis* fermented with inulin and individual β-glucans from barley, seaweed, and mushroom for 24 h were done in triplicate. Only 6 gels from two samples were compared pairwise at a time for the selection of the differentially expressed protein spots. This was because it was technically not feasible to compare all the 12 gels from the 4 different samples at the same time. As a result a total of 6 paired comparisons of the 2D-DIGE gels are shown in the following sections (3.2.1.1 to 3.2.1.6). The representative gel images of four treatments with spot labeled are shown in Figure 3.1-3.4. Spots with at least 1.5 fold change in normalized volume ratio were selected and considered as differentially expressed.

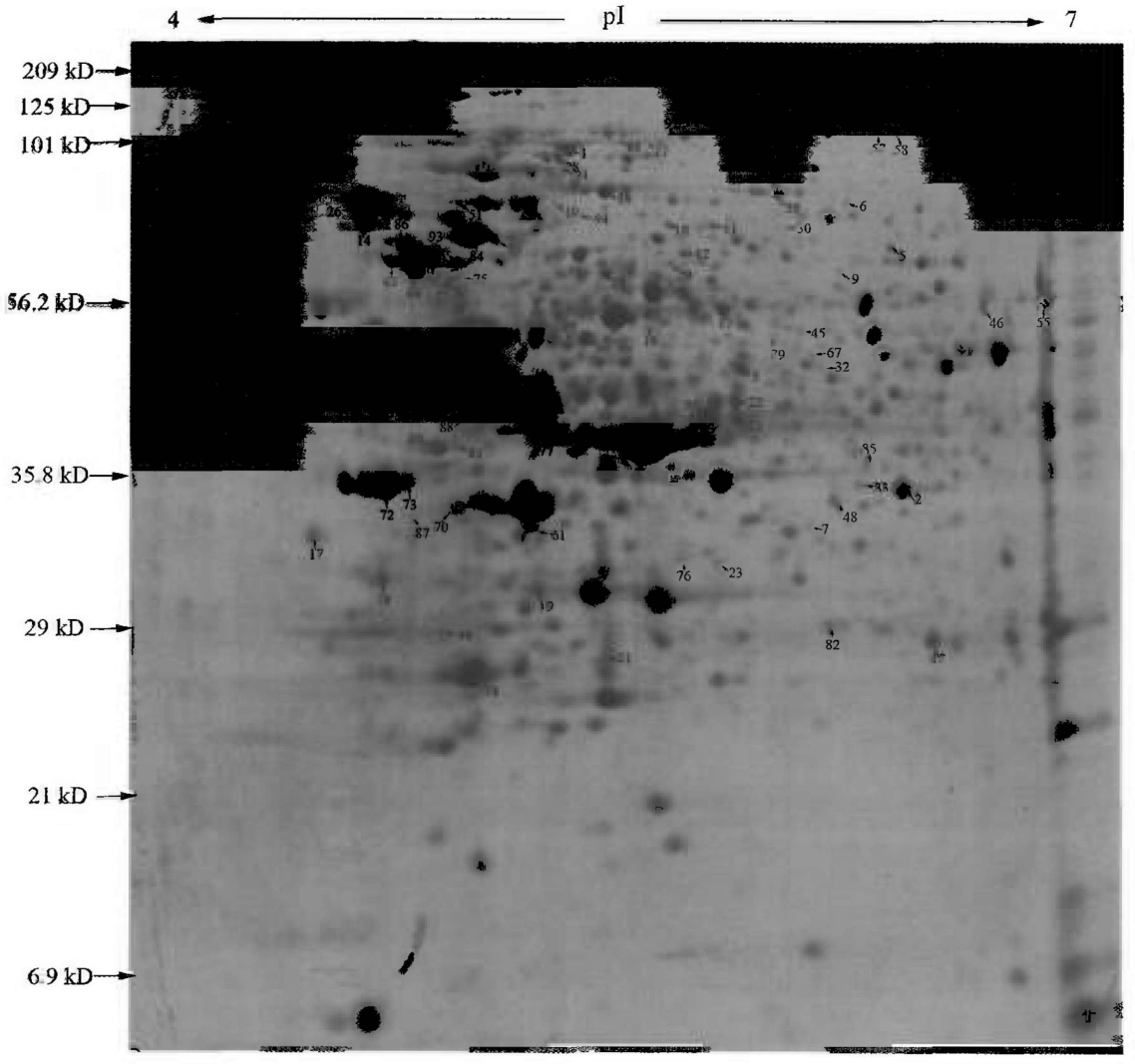

Fig. 3.1 Representative 2D-DIGE of total proteins from *B. infantis* treated with inulin. Identified spots with differentially expression, molecular weight and isoelectric point markers are denoted.

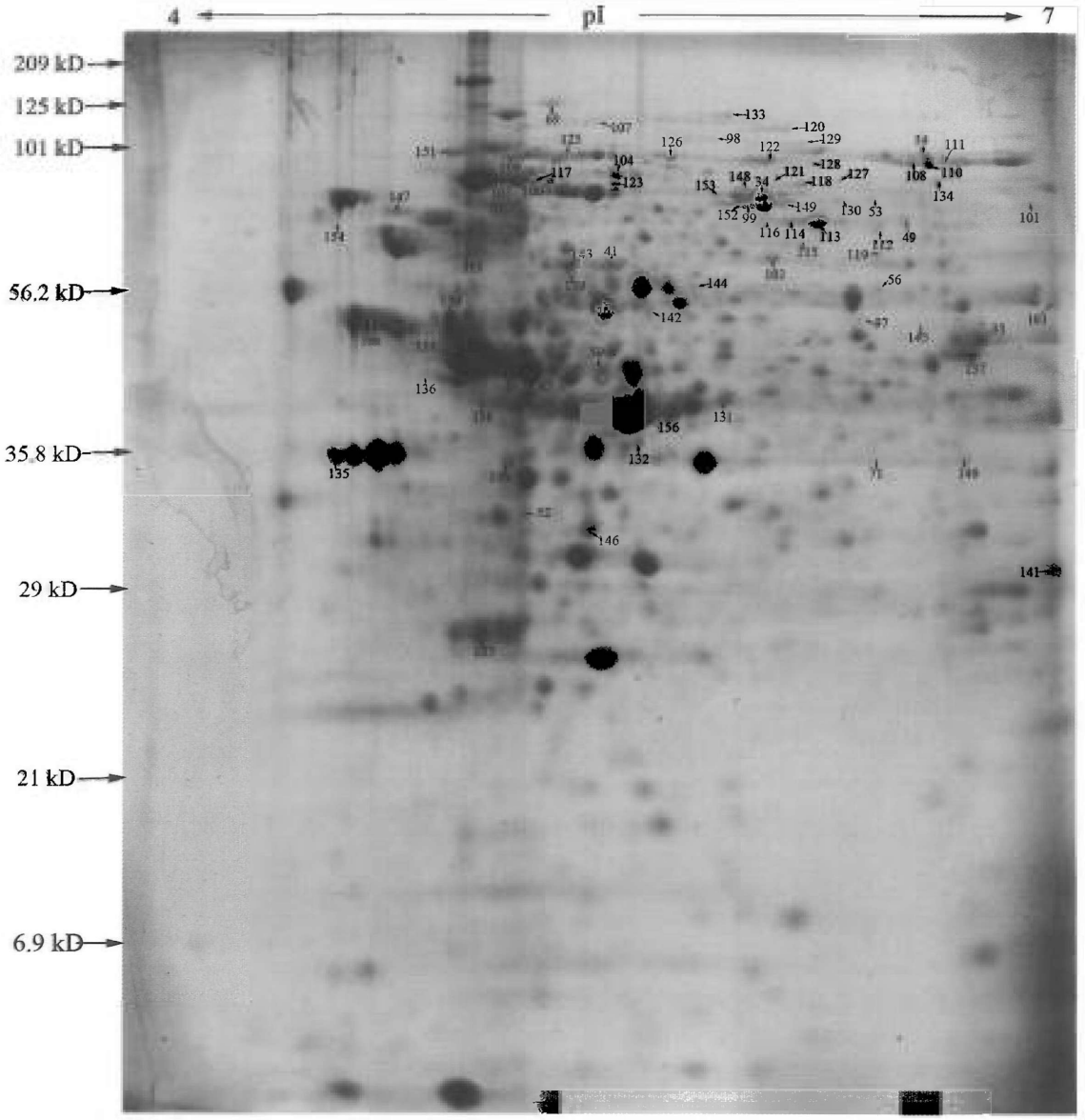

Fig. 3.2 Representative 2D-DIGE of total proteins from *B. infantis* treated with *β*-glucan from seaweed. Identified spots with differentially expression, molecular weight and isoelectric point markers are denoted.

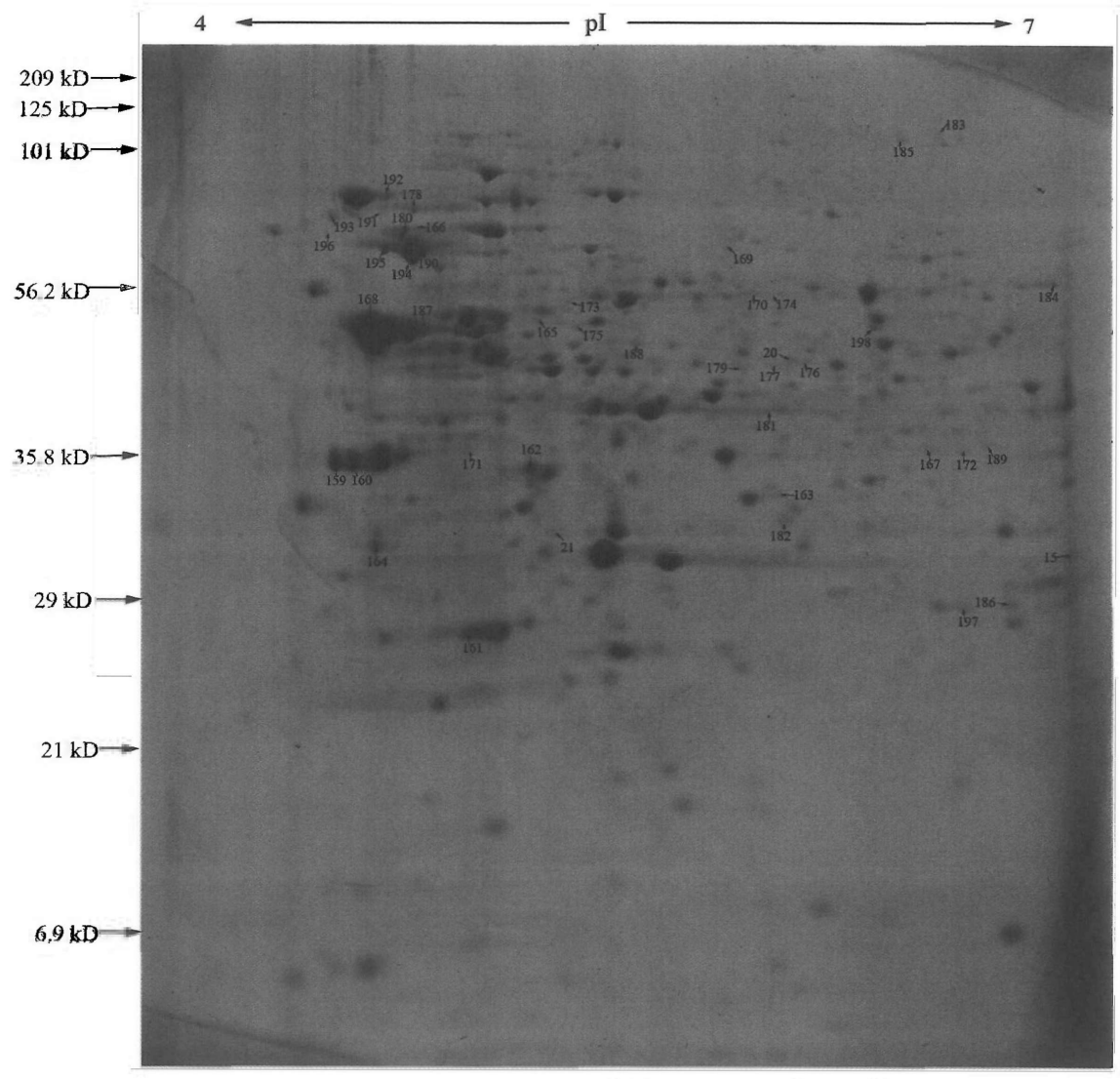

Fig. 3.3 Representative 2D-DIGE of total proteins from *B. infantis* treated with *β*-glucan from barley. Identified spots with differentially expression, molecular weight and isoelectric point markers are denoted.

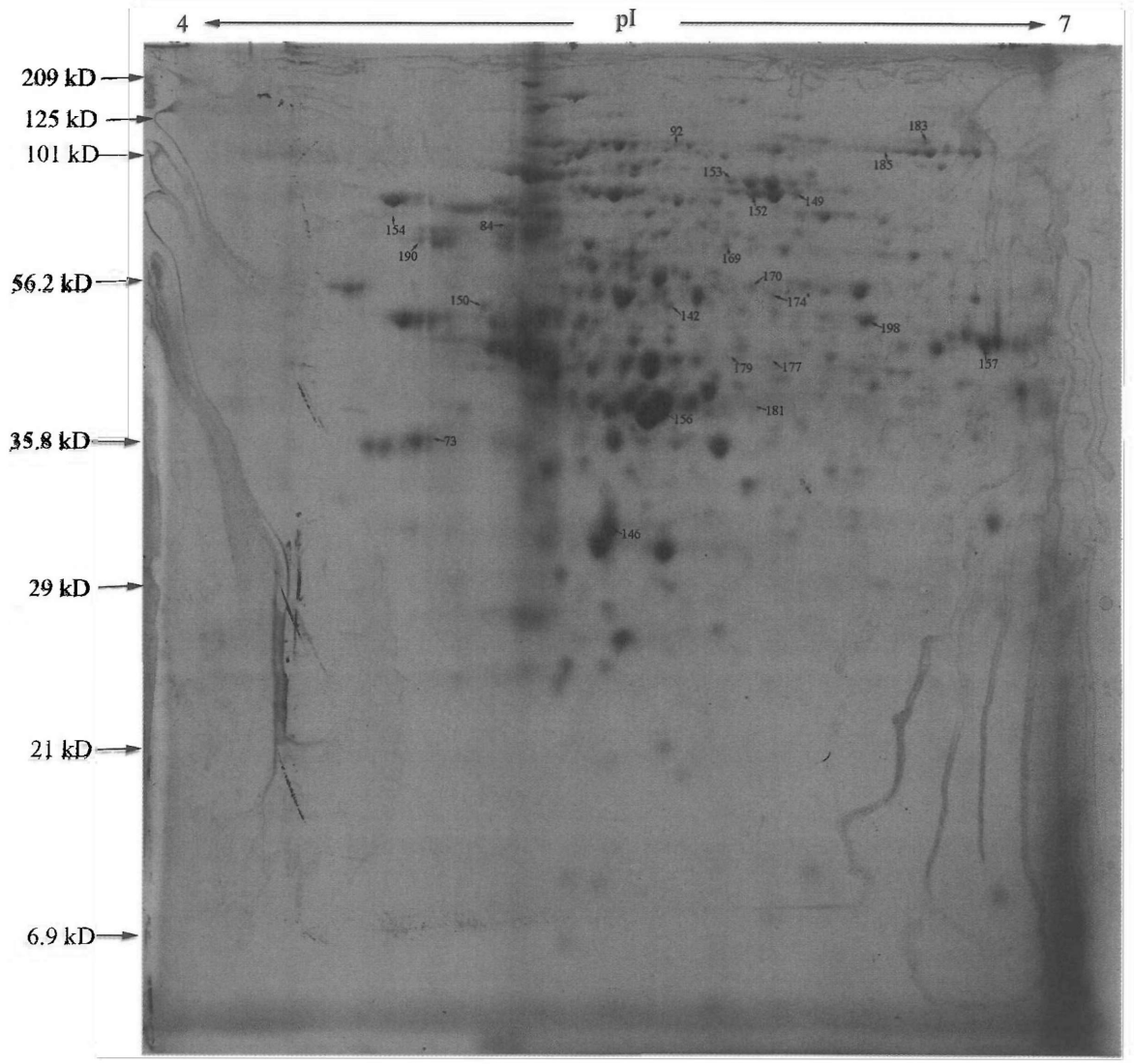

Fig. 3.4 Representative 2D-DIGE of total proteins from *B. infantis* treated with *β*-glucan from mushroom. Identified spots with differentially expression, molecular weight and isoelectric point markers are denoted.

3.2.1.1 Comparison between inulin and β-glucan from barley

By comparing the proteins from bifidobacteria cells incubated separately with inulin and barley in the 2D-DIGE gels, a total of 33 differentially abundant protein spots were identified. Thirty three unique proteins were identified by MALDI-TOF MS/MS with their information listed in Table 3.6. Among them, most proteins were only identified on the inulin gels and not on the barley gels while only 5 proteins appeared on barley gels.

Regarding carbohydrate metabolism, phosphoketolases (spot 1) play a key role in the unique pathway for hexose metabolism specifically in the genus *Bifidobacterium*, which could be used for identification of this genus. So far, two types of phosphoketolases have been discussed. The first type is specific for fructose-6-phosphate substrate and the second one could act on dual substrates: fructose-6-phosphate and xylulose-5-phosphate (Sanchez et al. 2004) . Sanchez et al reported that in those strains which acquired resistance to high bile concentration, considerably higher activity of phosphoketolase was observed in membrane and intracellular extracts, which indicated the greater glucose consumption in these resistant strains. Takahashi *et al* (2010) determined the crystal structure of the phosphoketolase in *Bifidobacterium longum* which is thiamine diphosphate dependent and proposed a model for its substrate binding.

6-Phosphofructokinase (spot 2) is a kinase enzyme in glycolysis, which catalyzes the formation of fructose-1, 6-bisphosphate from fructose-6-phosphate. Acetate kinase (spot 3) catalyzes the transformation of acetyl phosphate to acetate,

responsible for the formation of acetic acid during fermentation. Spot 4 and spot 8 were identified as the same protein: glucose-6-phosphate isomerase. These two spots together with pyruvate phosphate dikinase (spot 5), pyruvate kinase Ⅰ (spot 9) and transketolase (spot 10) are all involved in the glucose metabolism pathway: bifid shunt (Pokusaeva, Fitzgerald and van Sinderen 2011b).

Transketolase is a widely expressed enzyme in a wide range of organisms, whose structure in *Saccharomyces cerevisiae* and *Escherichia coli* was resolved already. Like phosphoketolase mentioned above, transketolase also requires thiamine diphosphate for catalytic activity in glucose metabolism (Takahashi et al. 2010). Vaugien et al (2002) chose pyruvate kinase gene for identification of bifidobacterium species in order to discriminate different species groups under this genus. Spot 6 was identified as ABC transporter related protein which might help the translocation of the β-glucan molecules from barley.

Spot 11 was identified as CTP synthase, which had been mentioned previously. The adaptation and response to bile salt at protein level in *Bifidobacterium animalis* subsp. *Lactis* (Sanchez et al. 2007). CTP synthase was proposed to be linked to the repair of DNA injuries during bile salt exposure. Bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase (spot 12) might be involved in nucleotide biosynthesis pathway. Changes of proteins that work on translation or transcription were also observed, and several proteins were found to be highly expressed in the presence of inulin including: 30S ribosomal protein S1 (spot 14), 30S ribosomal protein S2(spot 15), seryl-tRNA synthetase (spot

16), RNA polymerase factor (spot 17), transcription elongation factor (spot 32), and ribosome-binding factor (spot 33). This might indicate a modulation of protein synthesis in the presence of different NDCs. Spot 24 (bifunctional aconitate hydratase 2) and spot 25 (pyruvate dehydrogenase) might function in the energy metabolism process, while spot 28 (methionine synthase) spot 29, 30 (glycine dehydrogenase) might help amino acid metabolism. These proteins together may help the metabolism of NDCs through 'bifid shunt'. Both chaperone protein dnaK (spot 26) and protein clpB (spot 27) are important for bacterial stress response (De Dea Lindner et al. 2007), which might exert their functions in response to heat, acid, bile salt or other kinds of stress. In this case in the absence of simple sugars ready for use, these proteins might help utilize the structurally complicated inulin.

In this comparison, there were also several other proteins (spot 18, 19, 20, 21, 22, 23, 28, 29, 30) highly expressed in the presence of inulin, although so far no clear explanation could be linked to fermentation of inulin. It was obvious that *B. infantis* grew better in the presence of inulin, as many proteins were expressed at high level compared with those in the presence of barley. Inulin, as an oligosaccharide (MW about 5 kDa), is more readily uptaken and metabolized than barley β-glucan as a polysaccharide (MW 590 kDa), which is consistent with the fermentation data that the production of short chain fatty acids (SCFAs) as end metabolites in the presence of inulin was nearly twice as much as that in the presence of barley β-glucan (Table 3.5).

3.2.1.2 Comparison between inulin and β-glucan from seaweed

According to the data on fermentation analysis (Table 3.3), *B. infantis* exerted strong growth in the presence of both inulin and laminarin. The number of differentially expressed proteins which were isolated between the inulin and laminarin gels were 25 and 26, respectively between the two treatments, as compared to those in the comparison of inulin and barley β-glucan (3.2.1.1) in which most of the differentially expressed proteins were on the inulin gels only. A total number of 38 identified proteins in the inulin and seaweed gels are listed in Table 3.7.

Pyruvate-formate lyase (spot 34) is a key enzyme in the pyruvate-formate lyase (PFL) pathway, which is an endogenous NAD^+ regeneration process (Yamazaki et al. 2002). The expression of pyruvate-formate lyase in the bifidobacteria incubated with laminarin might indicate that the presence of laminarin would affect the PFL pathway metabolism, but this was not the case in the presence of inulin. Citrate synthase (spot 35) exists in almost all living cells and may provide precursors for some compounds during fermentation. Glyceraldehyde 3-phosphate dehydrogenase (spot 36) is an enzyme in the bifid shunt for glucose metabolism. It converts glyceraldehyde 3-phosphate to D-glycerate 1, 3-bisphosphate, which is further catalyzed to 3-phosphoglycerate by phosphoglycerate kinase (spot 42 and 43) with the production of ATP (Sela et al. 2008) . As mentioned above, glucose-6-phosphate isomerase (spot 37) could change glucose-6-phosphate into fructose-6-phosphate in the following steps for glucose metabolism. Spot 38 and spot 39 were identified as

pyruvate kinase which could transfer phosphoenolpyruvate to pyruvate together with the production of ATP. With the substrate fructose 1, 6-bisphosphate, fructose-1, 6-bisphosphate aldolase (spot 40) could catalyze the production of glyceraldehyde 3-phosphate in carbon metabolism. Acetyl-CoA synthetase (spot 41) may be involved in carbon catabolic pathway, and may participate in gene expression as well. Formate acetyltransferase (spot 44, 59) also help the formation of pyruvate through acetyl CoA (Knappe et al. 1974).

Spot 45 was one of the ATP binding proteins of ABC transporter for sugars. Bacterial ABC transporters are essential in many ways of bacterial life, for example, iron uptake, virulent defence and so on. Here, this specific sugar transporter should help the intake of inulin molecules from outside the bifidobacterial cells, and the high expression of this transporter might partly explain the high utilization efficiency of inulin by *B. infantis*. Spot 46 (putative oxidoreductase) and spot 47 (uridine phosphorylase) may be related to nucleotide transport and metabolism. Spot 49 (tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA), spot 50 (Chain A, Intact Elongation Factor from *E.Coli*) and spot 51 (prolyl-tRNA synthetase) may help the process of translation, among which spot 50 belongs to the GTP-binding elongation factors. Spot 52 (fabH 3-oxoacyl-(acyl carrier protein) synthase III) and spot 53 (fatty acid oxidizing complex) are involved in fatty acid metabolism and biosynthesis of laminarin. Five unique proteins from 6 spots (spot 54: 2-oxoglutarate dehydrogenase E1 component; spot 55: IMP dehydrogenase; spot 56: unnamed protein product / ATP synthase; spot 57 & 58: NADH: ubiquinone

oxidoreductase, chain G; spot 60: putative acetyltransferase) are proposed to help the production and/or conversion of energy. Stress protein DnaK (spot 62) appeared in the cells of both treatments, while its amount in the presence of inulin was three times that in the presence of laminarin. Hsp60 (spot 64) together with chaperonin GroEL (spot 63), which was also overproduced in the bile salt response of *B.longum* (Sanchez et al. 2005), were detected in larger amount in the presence of inulin. Spot 65 (trigger factor) may promote the folding of newly synthesized proteins. There are proteins belonging to other functional groups: amino acid metabolism (spot 66, 67 and 68); transcription (spot 69); secondary bile acid biosynthesis (spot 70); glycerophospholipid metabolism (spot 71) and spot 48 (ribose-phosphate pyrophosphokinase) was differentially expressed in this comparison.

3.2.1.3 Comparison between inulin and β-glucan from mushroom

Based on the fermentation results (in 3.1), inulin was fermented by the bifidobacteria better than the β-glucans from mushroom sclerotia of *Pleurotus tuber-regium* (PTR) in terms of pH drop, bifidobacteria proliferation and SCFA production. When comparing the gels from these two treatments, most differentially expressed proteins identified on the inulin gels could not be found on the PTR gels. A total number of 25 identified proteins are listed in Table 3.8.

Both spot 72 (outer membrane protein F) and spot 73 (chain A, Ompf porin mutant) might be constituent proteins of cell membrane porins which form channels on the membrane for the transportation of molecules. The larger amount of this kind

of proteins in the presence of inulin showed more active response of *B. infantis* to inulin. Triose phosphate isomerase (spot 74) is an important glycolytic enzyme catalyzing the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate which are intermediate products in glucose fermentative pathway, and triose phosphate isomerase links fucose metabolism and glucose metabolism through this conversion (Sela et al. 2008).

Several enzymes in the 'bifid shunt' pathway were found in this comparison: glucose-6-phosphate isomerase (spot 75); pyruvate kinase (spot 76); phosphoglycerate kinase (spot 77); transaldolase (spot 80, spot 81); polyphosphate glucokinase (spot 82); phosphopentomutase (spot 83); phospholgycerate mutase (spot 84) and phosphoglyceromutase (spot 90). Requena et al (2002) used transaldolase gene as a target for detection and enumeration of human bifidobacterium species and compared the efficiency with that of 16S rDNA sequence. ADP-glucose pyrophosphorylase (spot 79) is a protein in the glycogen biosynthesis, meaning that it is possible that inulin might affect glycogen biosynthesis to some extent. Another ABC transporter ATP-binding protein (spot 78) was found in the inulin gels. Spot 89 and spot 92 are both formate acetyltransferase which help the production of pyruvate. Spot 88 (ATP synthase subunit beta) and spot 91 (formate-tetrahydrofolate ligase) may work in energy production and conversion. Besides, several other proteins (spot 85, 86, 87, 93, 94, 95, 96) belonging to other functional groups were identified in the inulin gels.

3.2.1.4 Comparison between β-glucan from barley and from seaweed

The β-glucans from barley and laminarin from seaweed are β type glucans but are different in their sugar linkages: barley has a mixed β-1, 3 and β-1, 4 in the main chain while laminarin possesses a linear β-1, 3-linkage backbone with β-1, 6-branches (Table 3.1). Based on the fermentation results in 3.1 (Table 3.5), *B.infantis* produce similar amount of SCFA when incubated either with β-glucans from barley or with laminarin although the OMD for barley was only 16 % and that for laminarin was almost 37 % (Table 3.4). In this comparison, 38 proteins were found to be differently expressed, among which, 5 might be involved in the carbohydrate transport and metabolism (Table 3.9). ATP binding protein of ABC transporter for sugars (spot 97) and formate acetyltransferase (spot 99) were also identified in this comparison. The high expression of the 'ATP binding protein of ABC transporter for sugars (spot 97)' in the laminarin gels indicated that *B. infantis* could uptake the extracellular laminarin probably through the action of ABC transporter systems. Enolase (Spot 100) is an important enzyme in the bifid shunt, responsible for the conversion of 2-phosphoglycerate to phosphoenolpyruvate (PEP). Ruiz et al (2009) also reported that the membrane associated enolase also plays a role in bacterial colonization on the human gut. Glycogen phosphorylase (spot 101) belongs to a family of oligosaccharide phosphorylases and may be involed in glycogen metabolism. The other 32 proteins identified in this comparison (Table 3.9)

show no clear link to the fermentation at the present, so they are not discussed here.

3.2.1.5 Comparison between β-glucans from seaweed and mushroom

Compared with laminarin, PTR has a more complicated chain of with mixed glycosidic 1→3, 1→4 and 1→6 β-linkages (Table 3.1) and a lower solubility in water, which probably account for the low efficiency of fermentation (Table 3.4, 3.5).

There were 24 proteins identified in this comparison, all of which are listed in Table 3.10. Among them 4 might be involved in carbohydrate metabolism. Both spot 135 (Chain A, Ompf porin mutant in *E. coli*) and Spot 136 (Long-chain fatty acid outer membrane transporter in *Salmonella enterica*) are membrane proteins, which might help laminarin molecules passing through membranes or functioning in the binding of laminarin molecules in *B. infantis*. Spot 137 is triose phosphate isomerase, whose function has been discussed above (3.2.1.3). Fructose-1, 6-bisphosphate aldolase (spot 138) could convert fructose 1, 6-bisphosphate to glyceraldehyde 3-phosphate, which is an intermediate product in the central fermentative pathway.

The other 20 proteins (Table 3.10) might not affect the fermentation process in an obvious way according to their annotation and not discussed here.

3.2.1.6 Comparison between β-glucan from barley and mushroom

In this comparison, 40 proteins were found to be differentially expressed between Barley and PTR gels (Table 3.11). Two membrane proteins were identified: spot 159 (Chain A, Ompf porin mutant Y106f) and spot 160 (Chain A, Ompf porin

mutant D74a), together with one triose phosphate isomerase (spot 161). Spot 162 and spot 163 were identified the same as fructose-1, 6-bisphosphate aldolase. ABC transporter ATP-binding protein (spot 164) was highly expression in the presence of barley β-glucan instead of mushroom sclerotia. Two copies of enolase (spot 165 and spot 168) were found. Phosphotransferase system (PTS) enzyme I (Spot 166) belongs to the carbohydrate transport system 'phosphotransferase system PTS', which consists of enzyme II permease and HPr besides phosphotransferase enzyme I and is one of the several carbohydrate transport systems in *B. longum* NCC2705 (Parche et al. 2007). It was proposed the PTS in *B. longum* was complete glucose-specific and *B. infantis* may possess respective PTS specific for glucose and *N*-acetylglucosamine (Sela et al. 2008). By converting glucose to fructose-6-phosphate, the PTS incorporates glucose into central fermentative pathway. UDP-galactose-4-epimerase (spot 167), also called UDP-glucose-4-epinerase, functions in the final step of the 'Leloir pathway' (Sela et al. 2008) for galactose metabolism by producing UDP-glucose. Oligo-1, 6-glucosidase (spot 169) could break sucrose into fructose and glucose. The amount of oligo-1, 6-glucosidase in the presence of PTR was more than 3 times higher than that of barley (Table 3.11). Although no extra sucrose as carbon source was added into the fermentation medium, oligo-1, 6-glucosidase (spot 169) may help the degradation of the barley and PTR in the medium with unclear explanation so far. Glucose-6-phosphate 1-dehydrogenase (spot 170) acts in the pentose phosphate pathway transforming D-glucose 6-phosphate to D-glucono-1, 5-lactone 6-phosphate.

In the final steps of this pentose phosphate pathway (Sanchez et al. 2007), D-glucono-1, 5-lactone 6-phosphate is finally converted to ribose-5-phosphate or xylulose-5-phosphate, which could be inserted into the central fermentative pathway. This pentose phosphate pathway may act as an alternative to glycolysis for regulation of the intermediate compounds.

Table 3.7 A comparison of differentially expressed proteins in gels from *B. infantis* incubated with inulin and β-glucan from barley.

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Peptide Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change[a]	
												Inulin	Barley
	Carbohydrate transport and metabolism												
1	Putative phosphoketolase	*Bifidobacterium longum*	gi|23465532	92468.6	5.06	7	80	99.641				+∞	−∞
2	6-phosphofructokinase	*Geobacillus stearothermophilus*	gi|125125	34097.7	6.46	5	87	99.922				+∞	−∞
3	Acetate kinase	*Bifidobacterium longum*	gi|23465540	44172.5	5.47	11	197	100	EIADQYHIR	47	99.928	+∞	−∞
									TGDIDPAVVFHLIR	35	99.024		
4	Glucose-6-phosphate isomerase	*Bifidobacterium longum*	gi|23464881	62958.9	4.85	12	275	100	VASHAVLPYDQYLHR	53	99.981	+∞	−∞
									YSVDSAVGTSLAVVFGP	62	99.998		
5	Pyruvate phosphate dikinase	*Thermoanaerobacter tengcongensis*	gi|20807461	97378.8	5.2	7	75	98.917				+∞	−∞
6	ABC transporter related	*Bifidobacterium longum*	gi|213691817	73176.9	5.91	7	99	99.996				+∞	−∞
7	AccA acetyl-CoA carboxylase carboxyltransferase	*Escherichia coli*	gi|15799867	35219.3	5.76	6	103	99.998				+∞	−∞
8	Glucose-6-phosphate isomerase	*Bacillus subtilis*	gi|1934802	50495.5	5.04	3	108	100	SGTTTEPAIAFR	48	99.946	+∞	−∞
									TLSNEEGFESFVIPDDVG	45	99.894		
9	Pyruvate kinase I	*Escherichia coli*	gi|147276	50275.9	5.77	4	70	95.977	LNFSHGDYAEHGQR	38	99.701	+∞	−∞
10	Transketolase	*Bifidobacterium longum*	gi|23465296	75868	4.97	8	69	95.163				+∞	−∞
	Nucleotide transport and metabolism												

Table 3.7 (Continued)

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Peptide Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change[a]	
												Inulin	Barley
11	CTP synthetase	*Bifidobacterium longum*	gi|23465448	60945	5.4	4	71	97.152	ILFEEGLDAYVVR	30	98.097	+∞	−∞
13	Hypothetical protein pLG13_03	*Escherichia coli*	gi|32470118	58180.5	5.98	11	72	97.631				+∞	−∞
	Translation,ribosomal structure,biogenesis												
14	30S ribosomal protein S1	*Bifidobacterium longum*	gi|23465561	54600.1	4.68	11	138	100	QYLEETQSEVR	33	98.464	+∞	−∞
15	30S ribosomal protein S2	*Escherichia coli*	gi|15799851	26726.8	6.61	6	150	100	AGVHFGHQTR	46	99.953	−∞	+∞
									AVTLYLGAVAATVR	52	99.986		
16	Seryl-tRNA synthetase	*Bifidobacterium longum*	gi|23466184	47926	5.39	6	132	100	GTGFLNSHADEIYR	59	99.994	+∞	−∞
17	RNA polymerase factor sigma-32	*Caulobacter crescentus*	gi|16127328									1.0	3.8
	Unassigned												
18	Myosin-crossreactive antigen	*Bifidobacterium longum*	gi|23336666	70431.8	5.46	10	115	100				+∞	−∞
19	Flagellin B chain	*Bacillus thuringiensis*	gi|98515	29943.8	5.5	6	100	99.996				1.0	2.6
20	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	*Bacillus cereus*	gi|20146118	18369.3	5.31	4	115	100	**AAAALILAGLVSEGYTR**	**55**	**99.987**	−∞	+∞
21	Flagellin	*Bacillus thuringiensis serovar alesti*	gi|1588478	20544	6.28	4	104	99.999	SINSAADDAAGLAIATR	62	99.998	−∞	+∞
22	Acetylacetoin reductase	*Bacillus cereus*	gi|14475602	37696.7	5.43	6	138	100	LAGADYVLNPATQDVLAE	86	100	+∞	−∞
	Fatty acid metabolism, biosynthesis												
23	Enoyl-(acyl carrier protein) reductase	*Bacillus halodurans*	gi|15615406	28083.6	5.35	3	87	99.918	VNAISAGPIR	41	99.611	+∞	−∞

Table 3.7 (Continued)

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Peptide Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change[a]	
												Inulin	Barley
	Energy production and conversion												
24	Bifunctional aconitate hydratase	*Escherichia coli*	gi|15799802	93424.9	5.24	7	115	100	EALGLPHSDVFR	30	96.382	+∞	−∞
25	Pyruvate dehydrogenase	*Bacillus subtilis*	gi|16078522	41522.3	5.91	4	80	99.641	APAIFVVQNNR	61	99.998	+∞	−∞
Posttranslational modification,protein turn over,chaperones													
26	Chaperone protein dnaK	*Bacillus megaterium*	gi|118721	65210.7	4.69	8	207	100	FQLTDIPPAPR	50	99.965	+∞	−∞
									AVITVPAYFNDAER	64	99.998		
27	ClpB	*Bacillus anthracis*	gi|4127451	46165.3	5.17	6	75	98.813				+∞	−∞
	Amino acid transport and metabolism												
28	Methionine synthase II	*Bifidobacterium longum*	gi|23335057	85306.1	5.15	10	92	99.978				+∞	−∞
29	Glycine dehydrogenase	*Bacillus halodurans*	gi|15615377	54189.4	5.28	6	73	98.203	HEFVLSGR	40	99.728	+∞	−∞
30	Glycine dehydrogenase	*Shigella flexneri*	gi|24114156	104299.8	5.66	6	98	99.994	LQDAFPVLYTGR	38	99.398	+∞	−∞
	Transcription												
31	Stringent starvation proteinA	*Escherichia coli*	gi|15803763	24289.4	5.22	5	76	98.966	DSFLASLTEAER	34	98.542	+∞	−∞
32	Transcription elongation factor NusA	*Bifidobacterium longum*	gi|23466164	38002	5.64	10	108	100				+∞	−∞
33	Ribosome-binding factor A	*Vibrio parahaemolyticus*	gi|28899229	15271.8	7.79	6	73	98.161				+∞	−∞

[a] +∞ expressed in the gels of this treatment; −∞ not found in the gels of this treatment; the numbers represent fold change of Vol % on 2D-DIGE;

Table 3.8 A comparison of differentially expressed proteins in gels from *B. infantis* incubated with inulin and β-glucan from seaweed.

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Pep. Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change[a]	
												Inulin	Lam[b]
	Carbohydrate transport and metabolism												
34	Pyruvate-formate lyase	*Haemophilus influenzae*	gi|46128957	86244.2	5.75	6	96	99.99	SGVLTGLPDAYGR	44	99.799	−∞	+∞
35	Type II citrate synthase	*Shigella flexneri*	gi|24112004	48044.9	6.32	10	219	100	YSIGQPFVYPR	40	99.509	−∞	+∞
									ITFIDGDEGILLHR	45	99.85		
36	Glyceraldehyde 3-phosphate dehydrogenase	***Bifidobacterium longum***	gi|23465924	37693	5.23	11	254	100	YDSTHGTFR	39	99.491	4.6	1.0
									DDTTPTVVFGVNHDILK	39	99.482		
37	Glucose-6-phosphate isomerase	*Bifidobacterium longum*	gi|23464881	62958.9	4.85	7	89	99.956				+∞	−∞
38	Pyruvate kinase	*Bifidobacterium longum*	gi|23465557	55233.2	5.49	11	155	100	ISGYATDHGFDR	32	97.236	3.2	1.0
39	Putative pyruvate kinase	*Lysinibacillus sphaericus*	gi|14289139	47394.8	5.06	2	99	99.995	LNFSHGSHEEHAGR	91	100	1.0	2.3
40	Fructose-1,6-bisphosphate aldolase	*Bacillus subtilis*	gi|252453	2885.4	5.91	4	71	97.085				+∞	−∞
41	Acetyl-CoA synthetase	*Escherichia coli*	gi|15804661	72064.2	5.61	11	113	100				−∞	+∞
42	Phosphoglycerate kinase	*Bacillus halodurans*	gi|15616121	42048.8	5.04	1	69	95.689	ALSNPERPFTAIIGGAK	64	99.999	−∞	+∞
43	Phosphoglycerate kinase	*Bacillus halodurans*	gi|15616121	42048.8	5.04	3	101	99.997	ALSNPERPFTAIIGGAK	85	100	−∞	+∞
44	Formate acetyltransferase	*Haemophilus influenzae*	gi|16272145	86476.2	5.75	10	137	100	YPQLTIR	40	99.686	1.0	3.2
									SGVLTGLPDAYGR	42	99.796		
45	ATP binding protein of ABC transporter for sugars	*Bifidobacterium longum*	gi|23465255	40702.3	5.85	7	147	100	AEVVFDHVTR	38	99.522	+∞	−∞
									AAEILDLTEYLDR	56	99.992		

Table 3.8 (Continued)

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Pep. Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change[a]	
												Inulin	Lam[b]
59	Formate acetyltransferase	*Haemophilus influenzae*	gi|16272145	86476.2	5.75	10	162	100	YPQLTIR	50	99.973	1.0	3.1
									SGVLTGLPDAYGR	67	99.999		
	Nucleotide transport and metabolism												
46	Putative oxidoreductase	*Escherichia coli*	gi|12518884	49367.9	6.28	8	156	100	VPYEVGR	35	98.308	1.0	3.6
47	Uridine phosphorylase	*Escherichia coli*	gi|16131680	27141.8	5.81	7	147	100	SIGATTHVGVTASSDTFY	47	99.943	$+\infty$	$-\infty$
	Translation,ribosomal structure,biogenesis												
49	tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA	*Bacillus subtilis*	gi|16081153	69709.2	6.34	9	85	99.884	HQIFLEPEGR	37	99.587	$-\infty$	$+\infty$
50	Chain A, Intact Elongation Factor	*Escherichia Coli*	gi|4699821	43155.3	5.3	11	170	100	AGENVGVLLR	37	98.957	$-\infty$	$+\infty$
51	Prolyl-tRNA synthetase	*Bifidobacterium longum*	gi|23466273	66112.5	4.84	10	71	96.948				$+\infty$	$-\infty$
	Fatty acid metabolism, biosynthesis												
52	FabH 3-oxoacyl-(acyl carrier protein) synthase III	*Escherichia Coli*	gi|15801208	33494	5.08	4	89	99.948				$-\infty$	$+\infty$
53	Fatty acid oxidizing complex	*Escherichia coli*	gi|145900	79628.5	5.92	8	101	99.997				$-\infty$	$+\infty$
	Energy production and conversion												
54	2-oxoglutarate dehydrogenase E1 component	*Shigella flexneri*	gi|24111998	104980.5	6.08	14	184	100				$-\infty$	$+\infty$
55	IMP dehydrogenase	*Bacillus cereus*	gi|6561887	55357.9	6.35	6	109	100	GPLADTVHQLVGGLR	**41**	**99.678**	$+\infty$	$-\infty$
56	ATP synthase	*Escherichia coli*	gi|42283	55292.9	5.93	13	150	100				$-\infty$	$+\infty$
57	ubiquinone oxidoreductase	*Escherichia coli*	gi|145698290	100236	5.84	20	155	100				1.0	4.1

Table 3.8 (Continued)

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Pep. Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change[a] Inulin	Fold change[a] Lam[b]
58	NADH:ubiquinone oxidoreductase	*Escherichia coli*	gi|145698290	100236	5.84	27	459	100	HPLFVTNVDDTR	34	99.287	1.0	3.3
									IAPYYHLFGSDELSQR	38	99.701		
									LFETSENGLDYFTSVPAR	57	99.996		
									ELVGEENFYTGIAHGEQE	77	100		
									EIESYDAVLVLGEDVTQT	29	97.694		
60	Putative acetyltransferase	*Bacillus subtilis*	gi|16078014	16353.3	8.41	6	70	96.496				+∞	−∞
	DNA replication,recombination,and repair												
61	Excinuclease ABC subunit B	*Clostridium acetobutylicum*	gi|15893793	76006.2	5.32	9	74	98.47				+∞	−∞
	Posttranslational modification, folding and sorting												
62	Chaperone protein dnaK	*Bacillus megaterium*	gi|118721	65210.7	4.69	9	256	100	VIPNPEGNR	36	99.136	4.4	1.0
									FQLTDIPPAPR	48	99.943		
									AVITVPAYFNDAER	74	100		
63	Chaperonin GroEL	*Bifidobacterium longum*	gi|23464630	56802.7	4.72	13	286	100	IISYDEEAR	30	95.493	+∞	−∞
64	Hsp60	*Bacillus thuringiensis*	gi|15706393	42081.3	4.76	14	381	100	AQLEETTSEFDR	65	100	2.0	1.0
									QIAINAGLEGSVVVER	103	100		
									VASIVAEGDEATGINIVLR	76	100		
65	Trigger factor	*Bifidobacterium longum*	gi|23465520	49611.1	4.43	10	210	100	LTVTVEPEELNPYLDAAR	83	100	+∞	−∞
	Amino acid transport and metabolism												
66	Alanine dehydrogenase	*Staphylococcus aureus subsp. Aureus*	gi|21283381	40080	5.58	3	116	100	SLPLLSPMSEVAGR	41	99.719	8.5	1.0

Table 3.8 (Continued)

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Pep. Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change[a]	
												Inulin	Lam[b]
									HGVVHYAVANMPGAVPR	56	99.992		
67	Putative endopeptidase Clp ATP-binding chain C	*Streptococcus pyogenes*	gi|15675834	90657.4	6.42	6	70	96.576				+∞	−∞
68	Glycine dehydrogenase	*Escherichia coli*	gi|15833028	104280.8	5.62	7	70	96.245				1.0	3.2
	Transcription												
69	Polymerase beta, RNA	*Escherichia coli*	gi|223297	150523.3	5.15	24	169	100				−∞	+∞
	Secondary bile acid biosynthesis												
70	Choloylglycine hydrolase	*Bifidobacteirum longum*	gi|23465372	35102.5	4.71	3	102	99.998	FSDDEGNTYFGR	39	99.745	+∞	−∞
									NFDSVDEVEEALR	36	99.525		
	Glycerophospholipid metabolism												
71	Glycerol-3-phosphate cytidylyltransferase	*Oceanobacillus iheyensis*	gi|23100363	15542.9	6.13	4	70	96.654				−∞	+∞
	Unassigned												
48	Ribose-phosphate pyrophosphokinase	*Corynebacterium ammoniagenes*	gi|24418487	34814.5	5.72	12	399	100	LVANLLETAGATR	76	100	1.0	2.3
									DIVIVSPDHGGVTR	95	100		
									TINIVIPYYGYAR	40	99.721		
									FSDGEVQINIEESIR	70	100		

[a] +∞ expressed in the gels of this treatment; −∞ not found in the gels of this treatment; the numbers represent fold change of Vol % on 2D-DIGE;

[b] Lam: represents Laminarin;

Table 3.9 A comparison of differentially expressed proteins in gels from *B. infantis* incubated with inulin and β-glucan from mushroom.

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Pep. Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change [a]	
												Inulin	PTR
	Carbohydrate transport and metabolism												
72	Outer membrane protein F	*Escherichia coli*	gi|26246956	39330.1	4.78	4	150	100	YDANNIYLAANYGETR	130	100	+∞	−∞
73	Chain A, Ompf Porin Mutant Y106f	*Escherichia coli*	gi|14488510	37045.8	4.64	6	194	100	YADVGSFDYGR	45	99.938	2.8	1.0
									YDANNIYLAANYGETR	113	100		
74	Triose phosphate isomerase	*Bacillus cereus*	gi|20146166	15235.6	5	4	86	99.914	VGYVVLGHSER	51	99.982	+∞	−∞
75	Glucose-6-phosphate isomerase	*Bifidobacterium longum*	gi|23464881	62958.9	4.85	11	206	100	TFTTLETLTNAR	40	99.481	+∞	−∞
									VASHAVLPYDQYLHR	49	99.935		
76	Pyruvate kinase	*Vibrio cholerae*	gi|15640512	50407.4	5.79	6	74	98.605				+∞	−∞
77	Phosphoglycerate kinase	*Escherichia coli*	gi|15803460	41104.7	5.08	9	151	100	DYLDGVDVAEGELVVLENVR	69	100	+∞	−∞
78	ABC transporter ATP-binding protein	*Bacillus subtilis*	gi|15616033	29163.5	4.65	6	122	100	SGGPELAQR	35	99.14	+∞	−∞
									YLNEGFSGGEK	35	99.01		
79	ADP-glucose pyrophosphorylase	*Bifidobacterium longum*	gi|23335008	45803.2	5.48	7	203	100	AKPAVPFGGVYR	48	99.92	+∞	−∞
									FSPLLGSYVSPVPAQQR	32	97.215		
80	Transaldolase	*Bifidobacterium longum*	gi|23465295	39682.4	4.87	6	76	98.942	NATDIFR	29	98.547	+∞	−∞
81	Transaldolase	*Bifidobacterium longum*	gi|23465295	39682.4	4.87	8	87	99.918				+∞	−∞
82	Polyphosphate glucokinase	*Bifidobacterium longum*	gi|23464675	27233.3	5.7	8	228	100	YFNPDLFTVGGGVSR	116	100	+∞	−∞
83	Phosphopentomutase	*Bacillus halodurans*	gi|15614093	43562.2	5.28	2	88	99.933	HDYALKPFGR	33	99.008	+∞	−∞

Table 3.9 (Continued)

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Pep. Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change [a] Inulin	Fold change [a] PTR
									ISDIYDGEGVTESIR	48	99.969		
84	Phosphoglycerate mutase	*Bacillus subtilis*	gi|460258	56259.6	5.25	5	140	100	EAEFPGEER	30	96.704	11	1.0
									YPHVTFFMSGGR	87	100		
	Translation,ribosomal structure,biogenesis												
85	Phenylalanyl-tRNA synthetase	*Escherichia coli*	gi|15802126	36778.7	5.79	13	305	100	SFFENDLR	39	99.399	+∞	−∞
									NFFEEDLQIR	58	99.992		
									LAAETIDVSLPGR	34	97.99		
	Unassigned												
86	Hypothetical protein lp_2589	*Lactobacillus plantarum*	gi|28379117	34503.6	7.14	8	75	98.785				+∞	−∞
87	Autoinducer-2 (AI-2) modifying protein LsrG	*Pasteurella multocida*	gi|15603144	12252.1	5.55	7	72	97.462				+∞	−∞
	Energy production and conversion												
88	ATP synthase subunit beta	*Salmonella enterica*	gi|6625703	50279.8	4.9	10	281	100	DVLLFVDNIYR	62	99.998	+∞	−∞
									YTLAGTEVSALLGR	44	99.851		
									QLDPLVVGQEHYDTAR	54	99.985		
									NIAIEHSGYSVFAGVGER	30	96.838		
89	Formate acetyltransferase	*Bifidobacterium longum*	gi|23465524	90431	5.33	8	162	100	ILTGLPDAYGR	53	99.976	+∞	−∞
									YLDDNYLAVER	36	98.753		
90	Phosphoglyceromutase	*Oceanobacillus iheyensis*	gi|23099890	57142.8	4.92	4	99	99.995	YPHVTFFMSGGR	82	100	+∞	−∞
91	Formate--tetrahydrofolate ligase	*Bifidobacterium longum*	gi|23465070	53993.6	5.04	8	90	99.966	YPFHVQR	39	99.352	+∞	−∞

Table 3.9 (Continued)

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Pep. Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change[a]	
												Inulin	PTR
92	PflB	*Pasteurella multocida*	gi|15601940	87070.4	5.63	7	109	100	SGVITGLPDAYGR	52	99.968	1.0	2.5
	Posttranslational modification, Folding and sorting												
93	Hsp60	*Bacillus thuringiensis*	gi|15706393	42081.3	4.76	14	277	100	AQLEETTSEFDR	78	100	+∞	−∞
									QIAINAGLEGSVVVER	27	95.885		
									GFTTELDVVEGMQFDR	30	97.572		
94	Dihydroxy-acid dehydratase	*Bifidobacterium longum*	gi|23466332	66515.2	5.28	4	83	99.807	IAHQYYDDSDDSVLPF	52	99.975	+∞	−∞
95	S-adenosylmethionine synthetase	*Staphylococcus epidermidis*	gi|27468376	43965.5	4.95	6	184	100	IIVDTYGGYAR	39	99.424	+∞	−∞
									FVIGGPQGDAGLTGR	105	100		
96	Glycine cleavage system aminomethyltransferase T	*Shigella flexneri*	gi|24114158	40179.2	5.36	7	136	100	VPEGIGETAIVQIR	70	100	+∞	−∞

[a] +∞ expressed in the gels of this treatment; −∞ not found in the gels of this treatment; the numbers represent fold change of Vol % on 2D-DIGE;

Table 3.10 A comparison of differentially expressed proteins in gels from *B. infantis* incubated with β-glucans from barley and seaweed.

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Peptide Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change [a]	
												Barley	Lam [b]
	Carbohydrate transport and metabolism												
97	ATP binding protein of ABC transporter for sugars	*Bifidobacterium longum*	gi|23465255	40702.3	5.85	5	88	99.943	AAEILDLTEYLDR	39	99.307	$-\infty$	$+\infty$
98	Pyruvate-flavodoxin oxidoreductase	*Escherichia coli*	gi|16129339	128743.2	5.52	11	77	99.216				$-\infty$	$+\infty$
99	Formate acetyltransferase	*Haemophilus influenzae*	gi|16272145	86476.2	5.75	11	127	100	YPQLTIR	30	97.579	$-\infty$	$+\infty$
									SGVLTGLPDAYGR	49	99.966		
100	Enolase	*Staphylococcus epidermidis*	gi|27467479	47217.8	4.58	3	143	100	YNQLLR	30	96.803	3.1	1.0
									PIITDVYAR	35	98.839		
									ALVPSGASTGEHEAVEL	64	99.999		
101	Glycogen/starch/alpha-glucan phosphorylase	*Escherichia coli*	gi|213690992	90329.7	7.12	12	113	100	FNDGDFLR	26	96.093	1.0	2.9
	Nucleotide transport and metabolism												
102	CTP synthetase	*Escherichia coli*	gi|15803301	60336.1	5.63	18	274	100	QLYNAPTIVER	63	99.999	1.0	3.3
									GIAAASLAAILEAR	62	99.999		
103	Transcription termination factor Rho	*Escherichia coli*	gi|15804373	46967.5	6.5	9	75	98.866				$-\infty$	$+\infty$
	Translation,ribosomal structure,biogenesis												
104	Elongation factor G	*Escherichia coli*	gi|15803853	77532.5	5.24	12	296	100	IATDPFVGNLTFFR	61	99.998	1.0	2.4

Table 3.10 (Continued)

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Peptide Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change[a] Barley	Fold change[a] Lam[b]
									YDEAPSNVAQAVIEAR	39	99.697		
									EFNVEANVGKPQVAYR	69	100		
									VEVETPEENTGDVIGDLSR	44	99.896		
105	**Elongation factor G**	*Bacillus subtilis*	**gi|1644223**	**76496.5**	**4.82**	**11**	**230**	**100**	VNIIDTPGHVDFTVEVER	117	100	−∞	+∞
									VLDGAVAVLDAQSGVEPQTETVWR	48	99.965		
106	Elongation factor G	*Staphylococcus aureus*	gi|15923537	76564	4.8	5	69	95.163	VNIIDTPGHVDFTVEVER	40	99.65	−∞	+∞
	Energy production and conversion												
107	Carbamoyl Phosphate Synthetase	*Escherichis Coli*	gi|5821976	117768.3	5.22	13	78	99.418				−∞	+∞
108	Bifunctional acetaldehyde-CoA	*Listeria innocua*	gi|16800743	94651.9	6.48	5	74	98.361	**IGPEFHIPHGR**	**58**	**99.995**	1.0	11
109	Bifunctional acetaldehyde-CoA	*Listeria monocytogenes*	gi|16803674	94621.9	6.48	3	70	95.883	IGPEFHIPHGR	59	99.996	−∞	+∞
110	Bifunctional acetaldehyde-CoA	*Listeria innocua*	gi|16800743	94651.9	6.48	7	100	99.996	IGPEFHIPHGR	75	100	1.0	17
111	Oxoglutarate dehydrogenase	*Escherichia coli*	gi|43019	104979.6	6.04	11	118	100				−∞	+∞
112	Succinate dehydrogenase flavoprotein subunit	*Escherichia coli*	gi|15800427	64363.1	5.91	8	114	100	LGGNSLLDLVVFGR	27	95.073	−∞	+∞
									AAGLHLQESIAEQGALR	40	99.717		
113	Succinate dehydrogenase flavoprotein subunit	*Escherichia coli*	gi|15800427	64363.1	5.91	17	453	100	LRPAFPPK	36	99.474	1.0	3.2
									LPGILELSR	68	100		

Table 3.10 (Continued)

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Peptide Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change [a]	
												Barley	Lam [b]
									LGGNSLLDLVVFGR	70	100		
									AAGLHLQESIAEQGALR	107	100		
114	Succinate dehydrogenase flavoprotein subunit	*Escherichia coli*	gi|15830002	64381.1	5.85	15	386	100	LRPAFPPK	36	99.541	1.0	2.3
									LPGILELSR	51	99.985		
									LDDTSSEFNTQR	38	99.738		
									LGGNSLLDLVVFGR	42	99.877		
									AAGLHLQESIAEQGALR	93	100		
115	Succinate dehydrogenase flavoprotein subunit	*Bacillus halodurans*	gi|15615654	65377.6	5.49	6	71	97.217				−∞	+∞
116	Succinate dehydrogenase flavoprotein subunit	*Escherichia coli*	gi|15800427	64363.1	5.91	8	116	100	**AAGLH**LQESIAEQGALR	**31**	**98.478**	−∞	+∞
117	Bifunctional aconitate hydratase 2	*Escherichia coli*	gi|16128111	93439	5.24	18	359	100	EALGLPHSDVFR	58	99.997	−∞	+∞
									VEQAFELTDASAER	72	100		
									GFPLAYVGDVVGTGSSR	48	99.974		
									NPPAGEEEFLLDLLTNR	57	99.997		
118	Crystal Structure Of C418a	*Escherichia coli*	gi|6730181	85107.8	5.69	24	376	100	SGVLTGLPDAYGR	52	99.988	1.0	5.0
									TSTFLDVYIER	57	99.996		
									LAQFTSLQADLENGVNLEQTIR	47	99.961		
119	Pyruvate oxidase	*Escherichia coli*	gi|1009041	61507.2	5.81	10	157	100	APIVHALR	44	99.876	−∞	+∞
									AGGYLTDGTELHDTNFAR	37	99.336		
120	Pyruvate carboxylase	*Bacillus cereus*	gi|4584148	110066.2	5.58	13	194	100	VLYLEFNGQPR	55	99.989	−∞	+∞

Table 3.10 (Continued)

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Peptide Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change[a] Barley	Fold change[a] Lam[b]
									HIEVQILADEEGNVVHLYER	33	98.307		
121	Crystal Structure Of C418a	*Escherichia coli*	gi|6730181	85107.8	5.69	19	239	100				−∞	+∞
122	Aconitate hydratase	*Shigella flexneri*	gi|24112675	97685.9	5.65	19	241	100	FGDDEAFEENVR	60	99.998	1.0	3.8
									FVEFYGDGLDSLPLADR	54	99.992		
123	Phosphate acetyltransferase	*Escherichia coli*	gi|16130232	77123.9	5.28	15	296	100	LSVFKPIAQPR	64	99.999	−∞	+∞
									DAEVVLVEGLVPTR	56	99.995		
									HLNATIINEGDINTR	38	99.697		
124	**ATP synthase subunit beta**	*Salmonella enterica*	**gi|6625703**	**50279.8**	**4.9**	**15**	**345**	**100**	DVLLFVDNIYR	62	99.999	**2.3**	**1.0**
									VYDALEVQNGNER	43	99.888		
									LVLEVQQQLGGGIVR	44	99.912		
									QLDPLVVGQEHYDTAR	36	99.505		
125	Bifunctional aconitate hydratase 2	*Escherichia coli*	gi|15799802	93424.9	5.24	7	98	99.994				−∞	+∞
126	Pyruvate dehydrogenase E1	*Escherichia coli*	gi|15799798	99606	5.46	25	338	100	LHGYLPSR	34	98.844	−∞	+∞
									LTQEQLDNFR	33	98.534		
									LPYITFPEGSEEHTYLHAQR	34	98.928		
127	Crystal Structure Of C418a	*Escherichia coli*	gi|6730181	85107.8	5.69	11	142	100	SGVLTGLPDAYGR	28	95.045	−∞	+∞
	Amino acid transport and metabolism												
128	Glycine dehydrogenase	*Escherichia coli*	gi|15833028	104280.8	5.62	9	118	100				−∞	+∞
129	Isoleucyl-tRNA synthetase	*Escherichia coli*	gi|26245947	104302.6	5.62	17	158	100	AYEAYDFHEVVQR	39	99.735	−∞	+∞
130	Threonyl-tRNA synthetase	*Escherichia coli*	gi|16129675	73966.8	5.8	8	80	99.658				−∞	+∞
131	Aspartate Aminotransferase	*Escherichia coli*	gi|1311169	43497	5.63	10	242	100	NFGLYNER	44	99.888	1.0	2.6

Table 3.10 (Continued)

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Peptide Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change [a]	
												Barley	Lam [b]
									NYLGIDGIPEFGR	37	99.371		
									LREEFGVYAVASGR	36	99.333		
									ANYSNPPAHGASVVATILSNDALR	37	99.392		
132	Ornithine carbamoyltransferase	*Staphylococcus aureus*	gi|21284284	37738.8	5.15	3	75	98.785	MFDGIEFR	30	96.294	−∞	+∞
133	Trifunctional transcriptional regulator	*Escherichia coli*	gi|15801003	143679.3	5.58	16	79	99.569				−∞	+∞
	Unassigned												
134	ATP-dependent Clp protease	*Oceanobacillus iheyensis*	gi|23097548	90447.7	5.79	6	82	99.734				1.0	3.1

[a] + expressed in the gels of this treatment; − not found in the gels of this treatment; the numbers represent fold change of Vol % on 2D-DIGE;

[b] Lam: represents Laminarin;

Table 3.11 A comparison of differentially expressed proteins in gels from *B. infantis* incubated with β-glucans from seaweed and mushroom.

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Pep. Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change[a]	
												Lam[b]	PTR
	Carbohydrate transport and metabolism												
135	Ompf Porin Mutant Y106f	*Escherichia coli*	gi|14488510	37045.8	4.64	4	182	100	YADVGSFDYGR	30	97.404	+∞	−∞
									YDANNIYLAANYGETR	90	100		
136	Long-chain fatty acid outer membrane transporter	*Salmonella enterica*	gi|16761315	47691.4	4.9	5	71	97.018				+∞	−∞
137	Triose phosphate isomerase	*Bacillus cereus*	gi|20146166	15235.6	5	3	144	100	VGYVVLGHSER	123	100	+∞	−∞
138	Fructose-1,6-bisphosphate aldolase	*Geobacillus stearothermophilus*	gi|9297081	30798.8	5.47	3	89	99.954	SPVILGVSEGAAR	75	100	+∞	−∞
	Nucleotide transport and metabolism												
139	GMP synthase	*Escherichia coli*	gi|15832623	58627.9	5.24	9	248	100	HPFPGPGLGVR	47	99.906	+∞	−∞
									EFGYAQVEVVNDSALVR	41	99.64		
140	Guanosine 5'-monophosphate oxidoreductase	*Escherichia coli*	gi|15799788	37373.8	6.1	6	86	99.912				+∞	−∞
	Translation,ribosomal structure,biogenesis												
141	30S ribosomal protein S2	*Escherichia coli*	gi|15799851	26726.8	6.61	10	206	100	AGVHFGHQTR	65	100	+∞	−∞
									AVTLYLGAVAATVR	62	99.999		
142	Seryl-tRNA synthetase	*Escherichia coli*	gi|15800756	48353.5	5.34	13	212	100	EFDFEVR	35	99.407	1.0	2.2
									VLQLLGLPYR	46	99.953		
									AELDALQAEIR	36	99.544		

Table 3.11 (Continued)

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Pep. Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change[a] Lam[b]	Fold change[a] PTR
	Unassigned												
143	FeS cluster formation protein	*Bacillus subtilis*	gi|16080319	52696 1	5 14	6	90	99 961	DTDSALKENEDIFR	42	99 883	+∞	−∞
144	AhpF Alkyl hydroperoxide reductase subunit F	*Escherichia coli*	gi|15800321	57365 6	5 48	9	177	100	EAQSLLEQIR	34	98 196	+∞	−∞
145	Selenocysteine synthase	*Escherichia coli*	gi|15804132	50575 5	6 05	8	188	100	GELVEIGGAFR	33	97 916	+∞	−∞
									SAEVIQIQAQR	34	98 155		
									LPSAALTFTPHDGR	38	99 248		
146	Flagellin B chain	*Bacillus thuringiensis*	gi|98515	29943 8	5 5	9	271	100	EGGLNVGAR	38	99 641	1 0	3 7
									SINSAADDAAGLAIATR	142	100		
147	Hypothetical protein lin1928	*Listeria innocua*	gi|16800994	59803 3	4 56	4	89	99 952	GNSGVILSQLFR	70	100	+∞	−∞
	Energy production and conversion												
148	Bifunctional aconitate hydratase 2	*Escherichia coli*	gi|16128111	93439	5 24	5	80	99 658	EALGLPHSDVFR	31	97 94	+∞	−∞
149	Chain A, Crystal Structure Of C418a,C419a Mutant Of Pfl From E Coli	*Escherichia coli*	gi|6730181	85107 8	5 69	15	178	100	YPQLTIR	42	99 829	1 0	3 4
									SGVLTGLPDAYGR	46	99 924		
150	ATP synthase subunit beta	*Bacillus pseudofirmus*	gi|288554385	50900 3	5 01	14	304	100	YDDLPEDAFR	72	100	9 4	1 0
151	ATPases with chaperone activity	*Bifidobacterium longum*	gi|23335984	96921 6	4 86	22	197	100	LVQTEVGDQLAR	27	95 038	+∞	−∞
152	Formate acetyltransferase	*Vibrio cholerae*	gi|15641868	87728	6 01	10	133	100	YPQLTIR	37	99 38	1 0	5 2

Table 3.11 (Continued)

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Pep. Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change[a] Lam[b]	Fold change[a] PTR
									SGVLTGLPDAYGR	51	99.978		
153	Formate acetyltransferase	*Haemophilus influenzae*	gi|16272145	86476.2	5.75	7	134	100	YPQLTIR	39	99.449	1.0	3.5
									SGVLTGLPDAYGR	60	99.995		
	Posttranslational modification, folding and sorting												
154	Chaperone protein dnaK	*Bacillus megaterium*	gi|118721	65210.7	4.69	9	179	100	FQLTDIPPAPR	57	99.996	4.2	1.0
									AVITVPAYFNDAER	81	100		
155	GroEL	*Escherichia coli*	gi|18028158	51999.9	4.79	8	267	100	AAVEEGVVAGGGVALIR	99	100	+∞	−∞
									DTTTIIDGVGEEAAIQGR	101	100		
	Amino acid transport and metabolism												
156	Ornithine carbamoyltransferase	*Staphylococcus epidermidis*	gi|27469134	37828.1	6.09	3	130	100	VFDQAENR	59	99.999	1.0	2.1
									MFDGIEFR	61	99.999		
157	D-amino acid dehydrogenase	*Escherichia coli*	gi|170681298	47779.1	6.17	16	366	100	QGGTLQLFR	41	99.796	1.0	4.1
									TPAIPYEDLSVAR	86	100		
									DIAVLEDAGVPYQLLESSR	108	100		
	Transcription												
158	DNA directed RNA polymerase subunit alpha	*Yersinia pestis*	gi|22127885	36486.1	4.98	12	250	100	GFGHTLGNALR	35	99.131	+∞	−∞
									AEAIHYIGDLVQR	55	99.992		
									AATILAEQLEAFVDLR	28	95.5		

[a] +∞ expressed in the gels of this treatment; −∞ not found in the gels of this treatment; the numbers represent fold change of Vol % on 2D-DIGE;

[b] Lam: represents Laminarin;

Table 3.12 A comparison of differentially expressed proteins in gels from *B. infantis* incubated with β-glucans from barley and mushroom.

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Pep. Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change [a]	
												Barley	PTR
	Carbohydrate transport and metabolism												
159	Ompf Porin Mutant Y106f	*Escherichia coli*	gi|14488510	37045.8	4.64	12	479	100	VGGVATYR	49	99.978	+∞	−∞
									AVGLHYFSK	64	99.999		
									YADVGSFDYGR	114	100		
									YDANNIYLAANYGETR	133	100		
160	Ompf Porin Mutant D74a	*Escherichia coli*	gi|6729727	37017.8	4.69	13	433	100	VGGVATYR	37	99.641	3.2	1.0
									AVGLHYFSK	33	99.112		
									YADVGSFDYGR	89	100		
									YDANNIYLAANYGETR	135	100		
									TQDVLLVAQYQFDFGLRPSIAYTK	29	98.11		
161	Triose phosphate isomerase	*Bacillus cereus*	gi|20146166	15235.6	5	4	132	100	VGYVVLGHSER	102	100	+∞	−∞
162	Fructose-1,6-bisphosphate aldolase	*Geobacillus stearothermophilus*	gi|9297081	30798.8	5.47	3	88	99.947	SPVILGVSEGAAR	74	100	+∞	−∞
163	Fructose-1,6-bisphosphate aldolase	*Geobacillus stearothermophilus*	gi|9297081	30798.8	5.47	2	74	98.47	SPVILGVSEGAAR	63	99.999	+∞	−∞
164	ABC transporter ATP-binding protein	*Bacillus halodurans*	gi|15616033	29163.5	4.65	5	81	99.673	SGGPELAQR	53	99.995	+∞	−∞
165	Enolase	*Staphylococcus epidermidis*	gi|27467479	47217.8	4.58	4	96	99.991	PIITDVYAR	31	98.439	−∞	+∞
									ALVPSGASTGEHEAVELR	47	99.952		
166	PTS enzyme I	*Lactobacillus plantarum*	gi|50812224	63039.3	4.78	6	74	98.605	GFTTDIGGR	33	99.537	+∞	−∞

Table 3.12 (Continued)

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Pep. Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change [a] Barley	Fold change [a] PTR
167	UDP-galactose-4-epimerase	*Escherichia coli*	gi|15800468	37214.6	5.89	8	233	100	HPTFVEGDIR	86	100	+∞	−∞
									DSLAIFGNDYPTEDGTGVR	85	100		
168	Enolase	*Staphylococcus aureus*	gi|15923766	47087.8	4.55	5	232	100	YNQLLR	35	99.354	+∞	−∞
			gi|6015099						PIITDVYAR	44	99.913		
									ALVPSGASTGEHEAVELR	134	100		
169	Oligo-1,6-glucosidase	*Escherichia coli*	gi|146969	45240.1	5.5	9	124	100	GQTLLVIANLSR	27	97.902	1.0	3.6
170	Glucose-6-phosphate 1-dehydrogenase	*Salmonella enterica*	gi|16760836	55985.4	5.61	8	99	99.995	ETVLNLLALR	26	95.759	1.0	3.5
	Lipopolysaccharide biosynthesis												
171	ADP-L-glycero-D -mannoheptose-6-epimerase	*Escherichia coli*	gi|11513847	34879.1	4.8	7	297	100	FLFDEYVR	45	99.929	+∞	−∞
									YQAFTQADLTNLR	83	100		
									QILPEANSQIVGFR	55	99.993		
									EIPFLYASSAATYGGR	66	99.999		
	Nucleotide transport and metabolism												
172	Putative	*Bacillus subtilis*	gi|1146207	36314.6	6.09	3	115	100	LEIGDVAFITENR	103	100	+∞	−∞
	Translation,ribosomal structure,biogenesis												
173	Glycyl-tRNA synthetase	*Bacillus cereus*	gi|4584090	24742.4	8.28	6	107	100	NEITPGNFTFR	64	100	+∞	−∞
174	Glutamyl-tRNA synthetase	*Shigella flexneri*	gi|24113752	53862.9	5.65	16	147	100				1.0	3.3
175	Asparaginyl tRNA synthetase	*Escherichia coli*	gi|16128897	52537.4	5.17	19	277	100	IYTFGPTFR	43	99.898	+∞	−∞
									YGTVPHSGFGLGFER	88	100		

Table 3.12 (Continued)

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Pep. Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change [a]	
												Barley	PTR
176	Intact Elongation Factor	*Escherichia coli*	gi|4699821	43155.3	5.3	10	82	99.774				+∞	−∞
177	Tyrosyl-tRNA synthetase	*Escherichia coli*	gi|15802051	47983.3	5.49	10	90	99.965	GADLMQALVDSELQPSR	31	98.666	1.0	2.7
	Unassigned												
178	Glyceraldehyde kinase	*Enterococcus faecalis*	gi|29377570	60226.2	4.51	5	106	100	GNSGVILSQLFR	89	100	+∞	−∞
179	Capsid portal protein	*Salmonella enterica*	gi|16762248	39282.7	8.84	11	78	99.47				1.0	3.3
180	Hypothetical protein lp_2589	*Lactobacillus plantarum*	gi|28379117	34503.6	7.14	8	76	99.14				+∞	−∞
181	Hypothetical protein VV2_0983	*Vibrio vulnificus*	gi|27367378	44250.6	6.47	8	73	98.118				1.0	2.0
	Fatty acid metabolism, biosynthesis												
182	Enoyl-reductase	*Escherichia coli*	gi|26247623	27876.1	5.58	10	148	100	VNAISAGPIR	45	99.956	+∞	−∞
	Energy production and conversion												
183	2-oxoglutarate dehydrogenase	*Escherichia coli*	gi|15830005	104979.6	6.04	17	167	100				1.0	7.8
184	IMP dehydrogenase	*Bacillus cereus*	gi|6561887	55357.9	6.35	16	286	100	ENAQFIR	33	98.922	+∞	−∞
									ISGVPVVNNLDER	61	99.998		
									GPLADTVHQLVGGLR	60	99.998		
185	NADH:ubiquinone oxidoreductase	*Escherichia coli*	gi|145698290	100236	5.84	29	394	100	ADAVVVLENDLHR	76	100	1.0	4.0
									IAPYYHLFGSDELSQR	33	99.126		
									ELVGEENFYTGIAHGEQER	62	99.999		
186	Succinate dehydrogenase	*Escherichia coli*	gi|16128699	26752.2	6.32	8	156	100				+∞	−∞
187	ATP synthase subunit beta	*Salmonella enterica*	gi|6625703	50279.8	4.9	13	103	99.998				6.0	1.0
188	Adenylosuccinate Synthetase	*Escherichia coli*	gi|5542173	47184.2	5.24	13	213	100	TVLHLIPSGILR	56	99.995	1.0	3.3

Table 3.12 (Continued)

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Pep. Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change [a]	
												Barley	PTR
189	Guanosine 5'-monophosphate oxidoreductase	*Escherichia coli*	gi|15799788	37373.8	6.1	12	228	100	DILGGLR	62	99.998	+∞	−∞
									HVGGVAEYR	66	99.999		
	Posttranslational modification, folding and sorting												
190	Hsp60	*Bacillus thuringiensis*	gi|15706393	42081.3	4.76	13	400	100	AQLEETTSEFDR	78	100	6.3	1.0
									QIAINAGLEGSVVVER	114	100		
									VASIVAEGDEATGINIVLR	81	100		
191	Chaperone protein dnaK	*Bacillus megaterium*	gi|118721	65210.7	4.69	9	143	100	FQLTDIPPAPR	39	99.728	+∞	−∞
									AVITVPAYFNDAER	53	99.99		
192	Chaperone protein dnaK	*Bacillus megaterium*	gi|118721	65210.7	4.69	7	230	100	FQLTDIPPAPR	70	100	13	1.0
									AVITVPAYFNDAER	81	100		
									EQAITIKSSTGLSDDEIDR	56	99.996		
193	Chaperone protein dnaK	*Bacillus megaterium*	gi|118721	65210.7	4.69	12	240	100	FQLTDIPPAPR	64	99.999	+∞	−∞
									AVITVPAYFNDAER	102	100		
194	Hsp60	*Bacillus thuringiensis*	gi|15706393	42081.3	4.76	10	246	100	GFTTELDVVEGMQFDR	33	99.442	+∞	−∞
									VASIVAEGDEATGINIVLR	136	100		
195	Chaperonin GroEL	*Bifidobacterium longum*	gi|23464630	56802.7	4.72	16	342	100	IISYDEEAR	42	99.849	+∞	−∞
									NVVAGSNPIALR	66	99.999		
									VGQDGVVTVEDNNR	38	99.631		
									QIAENAGVSGDVVINTVR	61	99.998		

Table 3.12 (Continued)

Spot no.	Protein description	Organism	Accession no.	Theor. MW	Theor. pI	Pep. Count	Protein Score	Protein score CI%	Peptides identified by tandem MS	Ion Score	Ion CI%	Fold change [a]	
												Barley	PTR
	Transcription												
196	Transcription elongation factor	*Escherichia coli*	gi|15803711	54837.1	4.53	17	238	100	SGDFDTFR	28	96.412	+∞	−∞
									GVLYSVRPEAR	35	99.277		
197	Redox-sensing repressor Rex	*Bacillus subtilis*	gi|16077664	24076.6	6.66	7	99	99.995	LPLYYR	37	99.487	+∞	−∞
	Nitrogen metabolism												
198	Tryptophanase	*Escherichia coli*	gi|38704198	52755.6	5.88	15	154	100	GNFDLEGLER	32	99.083	1.0	2.7
									AVEIGSFLLGR	26	96.855		

[a] +∞ expressed in the gels of this treatment; −∞ not found in the gels of this treatment; the numbers represent fold of change of Vol % on 2D-DIGE;

3.2.2 Real-time RT-PCR analysis of proteins at transcriptional level

A total of 7 genes (Table 2.1) from 10 spots were selected in real-time reverse transcription polymerase chain reaction (Real-time RT-PCR). The genes coding those proteins that were expected to play an important role in carbohydrate transport and metabolism were tested for their changes at the transcriptional level in the fermentation of three types of β-glucans and the results are shown in Table 3.13. Bli 001 is the gene coding for an outer membrane porin protein, which may help the carbohydrate molecules to pass through bacterial cell membrane (Nikaido 2003). It was selected in the comparison of Barley and PTR, with its abundance in barley β-glucan gels 3.2 fold higher than that in the mushroom gels. It was not detected at the transcriptional level in the inulin gels, but was upregulated 2.9 fold in the seaweed gels and 1.4 fold in the mushroom gels (Table 3.13). Bli004 is the gene for another membrane protein which may be involved in the passage of carbohydrate molecules. The protein appeared on the gels of inulin and barley β-glucan treatments but not on the gels of mushroom (Table 3.9 and 3.12) which is in accordance with the fact that its transcription was not detected in mushroom β-glucan treatment (Table 3.13), and laminarin gave rise to 2.1 fold change compared to that by barley β-glucan at transcription level.

Bli 002 is the gene for ABC transporter related protein. This protein was identified in the inulin and barley β-glucan gels in the comparison of Inulin with PTR

gels and of Barley with mushroom, respectively. At protein level, it was expressed in the presence of inulin and barley β-glucan but not detected in mushroom β-glucan (Table 3.9 and 3.12). At transcription level, it was upregulated 2.6 fold in mushroom β-glucan culture and 0.93 fold in laminarin culture when compared with that in barley β-glucan culture. Although at the protein level, 'the ATP binding protein of ABC transporter' could not be identified in all the β-glucan systems due to the inherent limitations of the 2D-DIGE technique, the real-time RT-PCR study verified that *B. infantis* could use the ABC transporter systems for the uptake of all three kinds of β-glucans, with different fold of change in its expression levels (Table 3.13). Bli005 is coding for the ATP binding protein of another ABC transporter, and was down-regulated by seaweed and mushroom β-glucans. The expression of different ABC transporters in all the treatments indicated that *B. infantis* use diverse ABC transporters to different extent for each particular substrate. *B. infantis* genome contains many ABC transporter related genes, which may help to incorporate different kinds of nutrients into the cells for use (NCBI).

Bli003 coding for PTS enzyme Ⅰ， phosphotransferase. PTS system, is a characterized transport system in bifidobacteria (Yuan et al. 2006), and phosphotransferase is one of its components. Although PTS system enzyme Ⅰ protein was only detected in barley β-glucan culture (Table 3.12), its transcription was detected in all cultures. Laminarin gave a similar induction of 0.90 fold of that by barley β-glucan, but in the presence of mushroom β-glucan, the gene expression varied greatly among replicates with an average of 13 fold which might be due to the

unstable induction during fermentation process indicating that *B. infantis* may adopted the PTS system for carbohydrate transport more heavily when compared with the other β-glucan treatments.

Bli006 encodes for glycogen phosphorylase, which is an important enzyme responsible for sugar phosphorylation. This enzyme was identified in the comparison of Barley and Laminarin and the spot density is 2.9 fold upregulated by laminarin than that by barley β-glucan. The transcription for this enzyme is similar in all three β-glucan cultures: laminarin: 1.6; mushroom β-glucan: 1.4; barley β-glucan: 1.0. This may indicate that this phosphorylase may help *B. infantis* in the integral metabolism of different β-glucans to some extent.

Bli010 is the gene for transketolase, which is another important enzyme in the central fermentative pathway for glucose (bifid shunt) as mentioned before (3.2.1.1). Although only detected in the cells of inulin treatment, it was actually induced in all the treatments as confirmed by real-time RT-PCR analysis. The abundance at the transcript level was similar among different treatments, with laminarin: 1.2; mushroom β-glucan: 1.4; barley β-glucan: 1.0.

Table 3.13 Changes of identified proteins probably involved in β-glucan catabolism at transcription level by Real-Time RT-PCR

Spot no.	Protein description	Fold of change real-time RT-PCR		
		Seaweed[a]	Mushroom[a]	Barley[a]
160	Bli 001 outer membrane porin	2.9*	1.4	1.0[b]
78 and 164	Bli002 ABC transporter related	0.93	2.6*	1.0
166	Bli003 PTS system emzyme 1	0.90	13	1.0
72 and 159	Bli004 outer membrane protein F	2.1	nil	1.0
6 and 97	Bli005 ABC transporter ATP-binding protein	0.51*	0.44*	1.0
101	Bli006 glycogen phosphorylase	1.6	1.4	1.0
10	Bli 010 Transketolase	1.2	1.4	1.0

[a] Source of origin of β-glucans

[b] Normalized value for comparison with other values in the same row

* Significant difference compared with the normalized value of Barley β-glucan (ANOVA Tukey's multiple comparison test $P < 0.05$).

3.2.3 Hydrolytic enzyme activity for β-glucans during bifidobacterial fermentation

All of the cell-free fermentation broth and intracellular extracts from bifidobacterial cells collected after nearly 20 h fermentation were tested for glucanase activity and β-glucosidase activity. All the cell-free fermentation broth from different β-glucan systems did not display either glucanase activity or β-glucosidase activity (data not shown), which might indicate that *B. infantis* did not secrete these hydrolases into the culture medium. As for the intracellular activity, *B. infantis* exhibited 7.1 milli U/ml fermentation broth of glucanase activity in the presence of mushroom β-glucan and 4.1 milli U/ml in the presence of laminarin but no activity was detected for barley β-glucan from (Table 3.14). These might be due to different enzyme inducing ability of these β-glucans on the bifidobacteria. No β-glucosidase activity was observed intracellularly in all the treatments (data not shown).

There have been documented studies on the β-glucosidase activity assay of Bifidobacterium species. It was reported that three strains from *Lactobacillus* and *B. animalis* showed β-glucosidase activity which gave them the potential for the production of the biologically active aglycones in soymilk (Otieno, Ashton and Shah 2006). It was also reported that *B. longum* CRL849 produced β-glucosidase in the presence of raffinose, which was used as the principal energy source (Garro, Aguirre and Savoy De Giori 2006). Although β-glucosidase was detectable in general in the

genus *Bifidobacterium*, *B. infantis* did not exhibit any activity when encountering different sugars, including glucose, raffinose, lactose (Tsangalis et al. 2002), which was consistent with the present results.

Table 3.14 Intracellular glucanase activity of *B. infantis* fermented with β-glucans from different sources

β-glucan sources	**Glucanase activity of extracts**	**Glucanase activity before extraction in fermentation broth**
Seaweed	0.41±0.03 U/ml	4.1 milli U/ml fermentation broth
Mushroom	0.71±0.04 U/ml	7.1 milli U/ml fermentation broth
Barley	nil	nil

3.2.4 Proposed mechanistic model for utilization of *β*-glucans by *B. infantis*

Based on the identified proteins that were differentially expressed in different substrates and the changes of the important proteins at the transcription level together with the enzyme activity assay, a model for the metabolism of *β*-glucans from different sources by *B. infantis* is suggested and shown in Figure 3.5. The proteins identified in this study with different abundances among treatments are denoted in red.

The uptake and degradation of *β*-glucan molecules by *B. infantis* proceeded in a multistep manner: (1) binding of the *β*-glucan molecules on the cell surface; (2) internalization of the *β*-glucan molecules through the cell membrane by ABC type carbohydrate transporters or PTS proteins; (3) breakdown of *β*-glucan polymer into glucose or oligosaccharides by the action of *β*-glucan hydrolases; (4) incorporation of glucose into the central fermentative pathway 'bifid shunt'.

ATP-binding cassette transporters (ABC transporters) are a family of proteins which are universally present from prokaryotes to humans. In bacteria, ABC transporters could help mediate the import of nutrients into the cells, including ions, amino acids, peptides and sugars etc.. In gram-positive bacteria, the ABC-transporters for sugars are assembled by several protein components: a substrate binding protein anchored in the cell membrane exposed to the outside the cell specific for target carbohydrate molecules; two integral membrane proteins

responsible for the formation of the transport channel; the ATP binding protein as ATP hydrolyzing component (ATPase) for the energy source of the translocation (Bertram et al. 2004; Ehrmann et al. 1998). All the three types of β-glucans (laminarin, barley and mushroom) could be recognized and uptake through the ABC transporter systems by *B. infantis*, although the transport efficiency might be lower due to their larger chain length compared to that of inulin (MW 5000). The captured β-glucan molecules are translocated through the channel of the ABC assembly energized by ATP hydrolysis. It has also been reported that the utilization of the milk oligosaccharides through the ABC transporter complex in *B. infantis*, which underscores the importance of this family of transporters in the colonization of the infant GIT (Sela et al. 2008)

In *B. infantis*, β-glucan molecules were not only transported by ABC transporters, they were also channeled across the cell membrane through the phosphotransferase system (PTS). The PTS is involved in the transport and phosphorylation of a large variety of carbohydrates, both in gram-negative and gram-positive bacteria. The PTS is composed of the non-specific soluble proteins: enzyme Ⅰ (EⅠ) and histidine protein (HPr), and the carbohydrate specific component enzyme Ⅱ (EⅡ) (Postma, Lengeler and Jacobson 1993). The PTS catalyzes the carbohydrate phosphorylation coupled with its translocation through the cell membrane energized by the glycolytic intermediate phosphoenolpyruvate (PEP) (Postma, Lengeler and Jacobson 1993). One complete glucose-specific PTS in the genome of *B. longum* NCC2705 has been experimentally verified (Parche et al.

2007). According to the genome information, *B. infantis* possesses the PTS specific for glucose and N-acetylglucosamine (Sela et al. 2008) although the specificity needs further experimental verification. In this study, the proteomic and real time RT-PCR analysis revealed that the PTS could be used for the β-glucan substrate comprised solely of glucose units.

The real time RT-PCR analysis also showed a remarkable high transcription of PTS enzyme Ⅰ gene by the induction of β-glucan from mushroom (Table 3.12). As proposed by Postma et al (1993), the accumulation of carbohydrates by the non-PTS systems requires more than one ATP equivalent for both transport and phosphorylation on one monosaccharide unit while only one ATP is required by PTS system. This makes the PTS system a more favorable system for carbohydrate metabolism by anaerobic bacteria. The relatively lower solubility of mushroom β-glucan in water makes it more difficult to be used for the production of energy by *B. infantis* than seaweed and barley β-glucan. Therefore it was reasonable that the PTS might be adopted to a much larger extent with an average change fold 13 in RT-PCR (Table 3.12) by *B. infantis* when incubated with mushroom β-glucan in face of poor solubility of mushroom β-glucan.

The β-glucan molecules transported into the cell were predicted to be processed by intracellular carbohydrate hydrolases. Based on the glycoside hydolase classification system (www.cazy.org) and previous study (Sela et al. 2008), *B. infantis* has one glucan 1, 3-β-glucosidase (EC 3.2.1.58), which is capable of successive hydrolysis of β-D-glucose units from the non-reducing ends of

β-(1→3)-glucans, releasing glucose unit (http://www.enzyme-database.org/query.php?ec=3.2.1.58). The enzyme activity analysis (see 3.2.3) confirmed the expression of the active glucanase located intracellularly in the presence of laminarin and mushroom β-glucans. The documented study on β-(1→3)-glucan exo-hydrolase from *Euglena gracilis* demonstrated that this type of enzyme had a high specificity towards β-1, 3-glucosidic linkages and its action was not prevented by glucosyl substitution at the C-6 position on the β-1, 3-linked backbone (Barras and Stone 1969). In their substrate specificity test, seaweed β-glucan was efficiently hydrolyzed and led to the production of glucose and laminaribiose (Barras and Stone 1969) which is consistent with the result in this study that seaweed β-glucan strongly promoted the growth of *B. infantis*. On the other hand, it had been proposed that the low degree of hydrolysis of lichenin (mixed linkage of β-1, 3 and β-1, 4) with the production of glucose alone was ascribed to the breakdown of the terminal β-1, 3- linkages in the mixed-linked backbone (Barras and Stone 1969). This phenomenon was also observed in mushroom β-glucan with mixed β-1, 3 and β-1, 4 linkages, which was utilized by *B. infantis* at a rather slow rate (Table 3.4). The resulting glucose could be readily channeled into the 'bifid shunt' and the oligo residue, if remained, might be further degraded or exported as metabolites by the bifidobacterial cells. Further analysis of the detailed fate of the hydrolyzed residues is required.

Similarly, those β-glucan molecules phosphorylated and imported by the PTS may also be broken down by glucan 1, 3-β-glucosidase, to yield simple sugars which

are incorporated into the main fermentative pathway accordingly. It was also documented that a predicted β-1, 3- exoglucanase in *B. longum* NCC2705 belonging to the same group (EC 3.2.1.58) shared more than 50 % similarity to the characterized enzymes in yeast (Schell et al. 2002). The absence of glucanase activity in those bifidobacterial cells treated with barley β-glucan might be due to the high propotion of β-1, 4-linkages (nearly 75 %, see Table 3.1) in the backbone of this β-glucan, which might not efficiently induce the production of the glucanase. The detailed substrate specificity along with the enzymatic mechanism of the glucan 1, 3-β-glucosidase in *B. infantis* needs further experimental studies. The absence of β-glucosidase in *B. infantis* could be one of the reasons for the low rate of the degradation of all the β-glucans substrates in the fermentation analysis (see Table 3.4).

The final steps of the model are involved in the integration of simple sugars from the previous steps into the central fermentative pathway (Fig. 3.5). The glucose molecules released were phosphorylated by glucokinase into glucose-6-phosphate. The probable glucose-phosphate from the degradation of those β-glucans imported through the PTS could be transformed to glucose-6-phosphate by phosphoglucomutase. The glucose-6-phosphate from the above two sources could be isomerized by the action of glucose 6-phosphate isomerase (Spot 4, 8, 37,75) to β-D-fructose-6-phosphate, which is a key intermediate in the carbohydrate metabolic pathway 'bifid shunt' in bifidobaceria (Pokusaeva, Fitzgerald and van Sinderen 2011b; Sela et al. 2008).

As for inulin, intracellular inulinase activity was documented in *B. infantis* ATCC 15697 and several other *Bifidobacterium* species (Warchol et al. 2002). In the comparative proteomic study, ABC transporter for sugars and several enzymes in the 'Bifid shunt' were found highly expressed in the presence of inulin (Table3.7-3.9). It is reasonable to propose that the inulin molecules in the fermentation broth are imported into *B. infantis* cells by ABC transporters and broken down by inulinase, and the released fructose molecules are further degraded in the central fermentative pathway. The high expression of those enzymes involved in the fermentation demonstrated the high metabolism rate for inulin by *B. infantis* and this phenomenon is consistent with the fermentation results (Table 3.2-3.5) which showed the high fermentability and great capability as prebiotics.

A model for xylo-oligosaccharides (XOS) catabolism in *B. aninalis* subsp. *lactis* BB-12 has been proposed, which is more or less similar to this model and is comprised of substrate transport across the cell membrane by ABC system and degradation inside the cell (Gilad et al. 2010). On the other hand, an alternative model for XOS catabolism in *B. longum* subsp. *longum* has been proposed (Van Den Broek et al. 2008). *B. longum* possessed a multi-domain glycanase, which includes one transmembrane domain to anchor in the cell membrane and two carbohydrate binding modules as a catalytic domain to capture and degrade XOS substrate, with the hydrolyzed residues being transported into the cells. In fact, these two types of strategies for nutrient uptake adopted by *Bifidobacterium* species have been postulated previously (Palframan, Gibson and Rastall 2003).

The present study indicates the capability of *B. infantis* to ferment β-glucans with diverse chemical structures from different sources as primary carbon sources. It could be inferred from the information above that *B. infantis* is able to alter its metabolic patterns in face of the availability of different substrates (Pokusaeva, Fitzgerald and van Sinderen 2011b). Such flexibility in *B. infantis* provides a more favorable outcome for its own benefits under nutrient limited conditions, for the competitive colonization in human colon. The β-glucans from mushroom, notable for their ability to modulate the immune system, are gaining more and more attention as potential prebiotics. The proposed model for β-glucan catabolism by *B. infantis* is anticipated to broaden our understanding of how this type of NDC could be utilized by bifidobacteria and be classified as prebiotics.

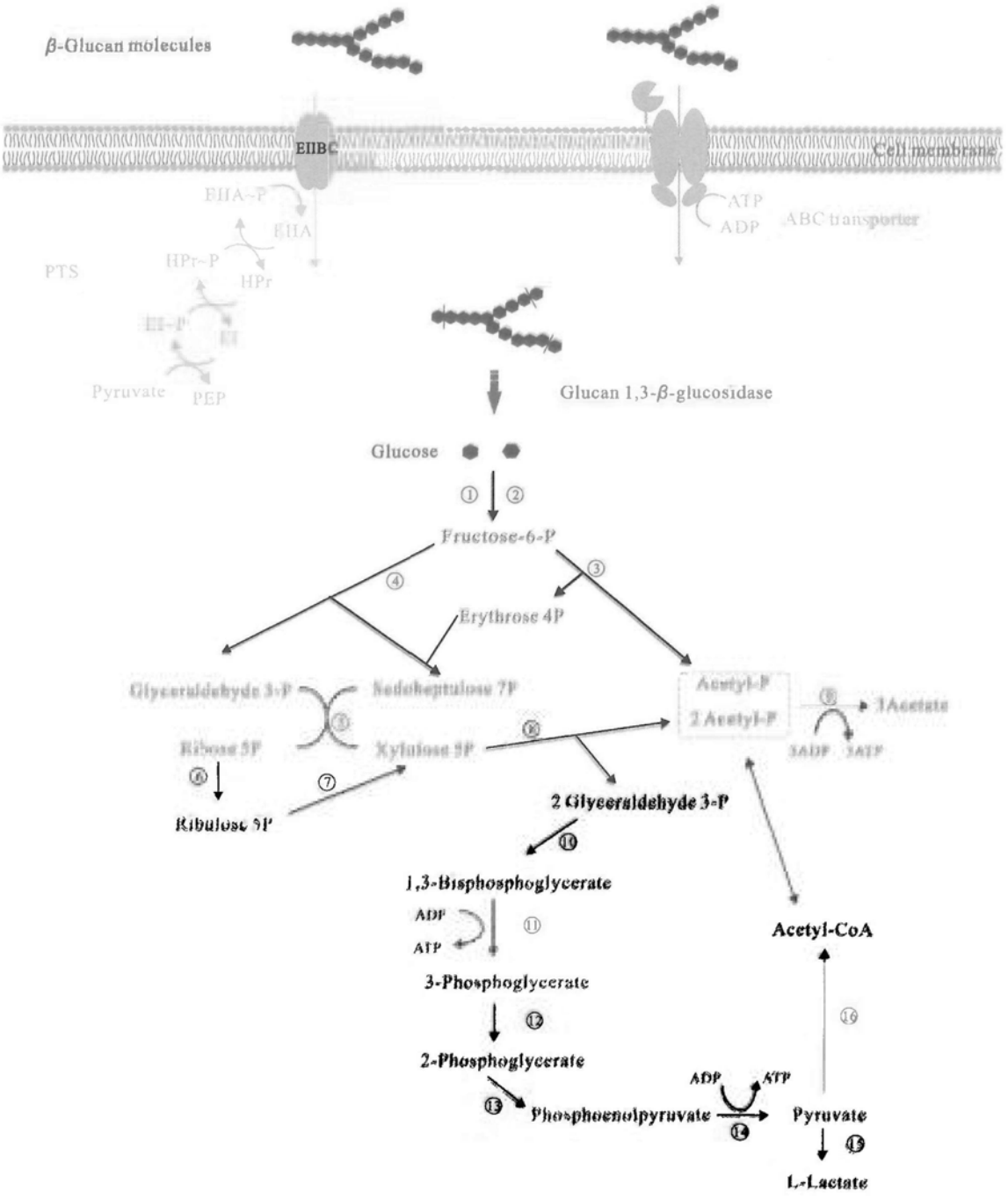

Fig. 3.5 Schematic representation of the proposed catabolic pathway of β-glucans by *B. infantis* based on the results of this study. Those enzymes involved in the carbohydrate catabolism are simplified by numbers. 1, glucokinase; 2, glucose-6-phosphate isomerase; 3, fructose-6-phosphate phosphoketolase; 4, transaldolase; 5, transketolase; 6, ribose 5-phosphate isomerase; 7, ribulose 5-phosphate epimerase; 8, xylulose-5-phosphate phosphoketolase; 9, acetate kinase; 10, glyceraldehyde-3-phosphate dehydrogenase; 11, phosphoglycerate kinase; 12, phosphoglycerate mutase; 13, enolase; 14, pyruvate kinase; 15, lactate dehydrogenase, 16, pyruvate formate lyase. Numbers circled in red denote the proteins identified in this study with different abundances among treatments.

Chapter 4 Conclusions

4.1 Concluding statements

The fermentation of four β-glucans (NDCs) from seaweed, barley, bacteria and mushroom by three *Bifidobacterium* strains, including *B. adolescentis*, *B. infantis* and *B. longum* was compared by a 24-h anaerobic batch system with inulin as control. Evaluation using parameters including pH, bacterial proliferation, organic matter disappearance (OMD) and SCFA production had indicated that inulin and β-glucan from seaweed had the best bifidogenic effect and were the preferred substrates for the bifidobacteria.

Based on the fermentation results, *B. infantis* was selected for a comparative proteomic study of its unique fermentation pattern to the different β-glucans. A total of 198 proteins were identified from spots with different abundances in the 2D-DIGE gels and some of them play an important role in carbohydrate catabolism, including ABC transporter, PTS protein and phosphoketolase. The changes at the transcriptional level of the genes coding these important proteins were verified by real-time RT-PCR analysis which gave a clear trend among all the β-glucans. The intracellular β-1, 3 glucanase activity was detected in *B. infantis* cells incubated with seaweed and mushroom β-glucans but not with barley β-glucan. A model was proposed for the metabolism of β-glucans by *B. infantis*.

In this study, it was demonstrated that the β-glucans from seaweed (laminarin) have a desirable bifidogenic effect that was comparable to the known prebiotic inulin

in terms of OMD and bacterial proliferation. Laminarin has the great potential to be commercialized as food ingredients and encapsulating materials due to its readily availability from seaweeds . The other bifidogenic material, mushroom β-glucans are well-known for their immunoregulation of the human immune system. Provided that the solubility of the mushroom β-glucans can be further improved, they are good candidates as functional food ingredients.

To our knowledge, this is the first study providing a comprehensive proteomic approach in *B. infantis* to reveal the underlying mechanism on the fermentation of β-glucans. There are only very few reports on the fermentation of β-glucans from different sources and very little knowledge on the molecular basis underlying the bifidogenic effects of β-glucans. This study for the first time reveals the possible degradation pathway of β-glucans by *B. infantis*, which is of great importance for the classification of these β-glucans as prebiotics and for their development as functional food products.

4.2 Future works

The present batch fermentation studies used only pure cultures of *Bifidobacterium* to evaluate the bifidogenic ability of the β-glucans from different sources. Since one of the criteria for prebiotics is selectivity, the β-glucans from different sources need to be fermented by human fecal inoculum *in vitro* to test whether they could be fermented by the human colonic microbiota in a selective way in future study. Animal models can also be adopted for the *in vivo* fermentation

analysis of these β-glucans from different sources, using rats as a possible model. The rats should be born germ-free and inoculated with human microbiota to imitate the conditions of human colon as much as possible, and the fecal samples as well as the physiological factors in the intestine of the rats should be monitored and analyzed after the ingestion of β-glucans. Regarding the structural complexity and low water-solubility of the β-glucans from mushroom sclerotia, some chemincal modifications might be made on the sugar chain or branching to increase its solubility in water in order to enhance their bifidogenic effect.

The hydrolytic specificity of the glucan β-1, 3 glucosidase from *B. infantis* needs more thorough analysis. The release of the residue from the hydrolysis of the β-glucans from different sources by the glucan β-1, 3 glucosidase from *B. infantis* could be monitored by HPLC or GC, so that the overall fate of the β-glucans from different sources could be followed. Besides, the glucan β-1, 3 glucosidase from *B. infantis* could be further fully characterized by the biochemical methods in enzymology.

A proposed mechanistic model has been made in this study, involving several proteins predicted to play important roles in the utilization of the β-glucans from different sources by *B. infantis*. Comparative proteome analysis on the membrane proteins of *B. infantis* would give a clearer verification of the transportation process adopted by the bacteria for the β-glucan molecules in the culture medium. The importance of the key proteins in the proposed model can be verified by molecular biology studies in bifidobacteria.

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Related publications

1. **Jin Yang Zhao**, Peter C. K. Cheung. Fermentation of β-glucans derived from different sources by bifidobacteria: evaluation of their bifidogenic effect. *Journal of Agricultural and Food Chemistry* 2011, 59, 5986-5992.
2. **Jin Yang Zhao**, P. C. K. Cheung. Fermentation of β-glucans derived from different origins by bifidobacteria. Abstracts of IFT Annual Meeting, Chicago, USA, 2010.