CZECH UNIVERSITY OF LIFE SCIENCES, PRAGUE,

FACULTY OF AGROBIOLOGY, FOOD AND NATURAL RESOURCES,

DEPARTMENT OF MICROBIOLOGY, NUTRITION AND DIETETICS

MSc. THESIS

Isolation of oligosaccharides from human, goat and sheep milk

MSc. Thesis
Submitted in partial fulfillment of the requirements
for the degree of master in Micobiological Sciences,
Czech University of Life Sciences, Prague, 2013

Supervisor of Diploma Thesis: **Prof. Ing. Vojtěch Rada, CSc.**
Consultant of Diploma Thesis: **Ing. Šárka Ročková, Ph.D**
Author of Diploma Thesis: **BSc. Mohamed Abd EL-wanis Ahmed**

Declaration

I declare that I have elaborated my diploma thesis "**Isolation of oligosaccharides from human, goat and sheep milk**" on my own with a help of literature listed in References.

This MSc. thesis is submitted in partial fulfillment of the requirements for the degree of master in Micobiological Sciences, Czech University of Life Sciences, Prague, 2013.

Prague, 2013.

In Prague date..... Signature

BSc. Mohamed Abd El-wanis Ahmed

.....

ACKNOWLEDGMENTS

I would like to express my deepest thanks to my mentor, the **wise, fairly-minded and honest, Prof. Ing. Vojtěch Rada, CSc**. Head of the Department of Microbiology, Nutrition and Dietetics, Faculty of Food and Natural Resources, Czech University of Life Sciences, Prague in the Czech Republic, for his guidance, helpful encouragement and continuous support during the practical stages of the experiments.

Similarly, I wish to express my special gratitude and appreciation to **Ing. Šárka Ročková, Ph.D,** for her sincere help, diligent effect, valuable ideas and remarks during the research work.

I would like to thanks colleague **Roman Švejstil** for help during my MSc. Work.

I wish to express my special gratitude and appreciation to **Ing. Hammad Ketta, Ph.D,** for his help and supporting me in my life.

I am grateful to **my parents** and **my wife** for their help and supporting me, during all stages of the research work.

I thank also all the **colleagues** and **technicians** of the Department of Microbiology, Nutrition and Dietetics for their assistance and friendship, that made me feel home.

ABSTRACT

Human milk oligosaccharides (HMOs) have specific biological functions. Such functions may include prebiotic activity, anti-adhesive activity, anti-inflammatory properties, and a role in brain development. HMOs serve as prebiotic compounds that can selectively stimulate the growth and activity of intestinal bacteria especially bifidobacteria that contribute health and well-being. *Bifidobacterium longum* can be found as a component of the gastrointestinal micro flora of breast-fed infants and adults that play an important role in the maintaining and promoting of human health by eliciting a number of beneficial properties. *Bifidobacterium longum* subspecies *longum* and *infantis* can utilize a diverse range of dietary carbohydrates and are able to grow on human milk oligosaccharides.

Our aims were to isolate oligosaccharides from human human, sheep and goat milk. Additional aims were to test the ability of *B. longum* ssp. *longum* and *B. longum* ssp. *Infantis* to grow in human milk and to utilize human milk oligosaccharides.

HMOs were isolated by using by gel-filtration chromatography (GLC) and screened the fractions by Thin-layer chromatography (TLC). Five strains of bifidobacteria of human origin and 2 strains of bifidobacteria of animal origin were tested for growth in milk samples by using microtiter plate technique.

Human milk selectively stimulated the growth of specific bifidobacterial strains. Bifidobacteria of human origin utilized HMOs in contrast with Bifidobacteria from animal origin. Growth of Bifidobacteria strains were accompanied by a decrease of pH. There were significant differences ($P < 0.05$) between bacterial counts of *B. bifidum* and *B. animalis* in milk samples tested.

Keywords: Probiotics, Prebiotics, Human milk oligosaccharides, Bifidobacteria.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF USED ABBREVIATIONS

HMOs	Human milk oligosaccharides
GOS	Galacto-oligosaccharides
IMOs	Isomalto-oligosaccharides
TGOS	Trans-galacto-oligosaccharides
DP	Degree of polymerization
GALT	Gut-associated lymphoid tissue
β-gal	β-galactosidase
SCFA	Short-chain fatty acids
GLC	Gel lequid chromatography
TLC	Thin-layer chromatography
Fig.	Figure
No.	Number
Tab.	Table
FOS	Fructo-oligosaccharides
XOS	Xylo-oligosaccharides
WHO	World health organization
FAO	Food and agriculture organization
IUB	International Union of Biochemistry
IUPAC	International Union of Pure and Applied Chemistry

1. INTRODUCTION

Probiotics and prebiotics play an important role in human nutrition. In recent years there has been a significant increase in research on the characterization and verification potential health benefits associated with the use of probiotic and prebiotic. The concept probiotic is defined by a United Nations and World Health Organization Expert Panel as "live micro-organisms which when administered in adequate amounts confer a health benefit on the host. Lilly and Stillwell (1965) defined probiotics as substances produced by one microorganism that promoted the growth of another microorganism. To improve and help the health of infant's which can not get mother's milk at born prebiotics has been an alternative. In the term prebiotic the preposition "pro" was exchanged for "pre" which means "before" and has been defined as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/ or activity of one or a limited number of bacteria in the colon (Gibson and Roberfroid., 1995). A more recent definition of the term is ''a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health'' (Gibson, *et al*., 2004). Main prebiotic oligosaccharides are: Galacto-oligosaccharides (GOS), Fructo-oligosaccharides (FOS), Isomalto-oligosaccharides (IMOS), Xylo-oligosaccharides (XOS) and human milk oligosaccharides (HMOs).

Human milk contains a high concentration of diverse soluble oligosaccharides that are carbohydrate polymers formed from a relatively small number of different monosaccharides.

Human milk, which nourishes the early infants, is a source of bioactive components for the infant growth, development and commensal formulation as well. Beefits of mother's milk is given by specific compounds known as human milk oligosaccharides. Feeding infants' breast milk of healthy mothers is associated with a lower incidence of infectious and allergic diseases. The amount of oligosaccharides in milk of most animal species is low compared with human milk. Although most mammalian milk contains oligosaccharides, oligosaccharides in human milk exhibit unique features in terms of their types, amounts, sizes, and functionalities. In addition to the prevention of infectious bacteria and the development of early immune system, human milk oligosaccharides are able to facilitate the healthy intestinal microbiota.

Bifidobacteria as a probiotic bacteria have been emerged on the food market for more than 10 years, and considered as important probiotics and used in the food industry to relieve

and treat many intestinal disorders. Bifidobacteria is gram-positive, non-motile, non-spore forming, anaerobic bacteria with irregular cell morphology. They are naturally found in the human gastrointestinal tract (GIT). They colonize the intestine of newborn children within the first few days after birth and in breast-fed infants represent up to 95% of the intestinal microflora. The most frequently detected species in the faeces of breastfed infants are *Bifidobacterium longum*, *Bifidobacterium breve*, and *Bifidobacterium bifidum*. The amount as well as species distribution of bifidobacteria changes depending on age.

Bifidobacteria exert a range of beneficial health effects, including the regulation of intestinal microbial homeostasis, the inhibition of pathogens and harmful bacteria that colonize and infect the gut mucosa, the modulation of local and systemic immune responses and absorption of minerals and vitamins.

2. REVIEW OF LITERATURE

Probiotics, synbiotics and prebiotics

2.1. Probiotics

The concept of probiotics emerged from observations early in the 19th century by Russian immunologist Elie Metchnikoff, who hypothesized that the long and healthy lives of Bulgarian peasants were rooted in their consumption of fermented milks containing beneficial *Lactobacillus*, and the positive effect of these microbes on colonic health (Dixon, 2002). The word ‘‘probiotics’’ was initially used as an antonym of the word ‘‘antibiotic’’. It is derived from Greek words **pro** and **biotic** and translated as ‘‘for life’’ as mintioned by Hamilton, *et al*., (2003). Lilly and Stillwell (1965) defined probiotics as substances produced by one microorganism that promoted the growth of another microorganism.

Parker (1974) was the first to use the term probiotic in the sense that it is used today, he defined probiotics as “organisms and substances which contribute to intestinal microbial balance’’. Fuller (1997) attempted to improve Parker's definition of probiotic as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Salminen, *et al*., (1998) defined probiotics as “foods which contain live bacteria which are beneficial to health", whereas Marteau, *et al*., (2002) defined them as “microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well being".

Some modern definitions include more acurately a preventive or therapeutic action of probiotics. Charteris, *et al*., (1997) defined probiotics as “microorganisms, which, when ingested, may have a positive effect in the prevention and treatment of a specific pathologic condition".

Currently probiotic is defined by a United Nations and World Health Organization Expert Panel as “live micro-organisms which when administered in adequate amounts confer a health benefit on the host” indeed FAO/WHO (2002).

2.2. Synbiotics

Synbiotics are mixtures of probiotics and prebiotics were firstly defined in 1995 by Gibson and Roberfroid. That mix would benefit the host by improving implantation and survival of the selected microbial supplements. The potential benefit of synbiotics is that they may increase both the gut delivery efficacy and the activity of the beneficial organism within the gut, although the evidence that they can actually achieve this is still not clear (Worthley *et al*., 2009). Because of the nutritional benefits associated with microbiota management approaches, foods are the main vehicle for probiotics, prebiotics and synbiotics. However, there may also be potential pharmaceutical applications, but till now most evidence for that is hypothetical.

2.3. Prebiotics

The term prebiotics was introduced by Gibson and Roberfoid (1995) as an alternative approach to the modulation of the gut microbiota, prebiotics have been used and these are defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon. A more recent definition of the term is ‘‘a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health’’ (Gibson, *et al*., 2004). Any dietary ingredients that can reach the colon have the potential of being a prebiotic. However, according to fulfil the criteria, it should be able to resist the digestion process, which involves gastric acids, intestinal brush border and pancreatic enzymes, and gastrointestinal absorption, and be selectively fermented by especific genera of colon bacteria (Lomax and Calder, 2009). Gibson, *et al*., (2004) observed that not all dietary carbohydrates are prebiotics, and obvious criteria need to be established for classifying a food ingredient as a prebiotic. These criteria are 1- Resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption. 2- Fermentation by intestinal microflora. 3- Selective stimulation of the growth and/or activity of those intestinal bacteria that contribute to health and well-being.

Resistance, in the first criterion, does not necessarily mean that the prebiotic is completely indigestible, but it should guarantee that a significant amount of the substance is available in the intestine (especially the large bowel) to serve as a fermentation substrate. Although each of these criteria is important, the third one is the most difficult to fulfill.

2.3.1. Prebiotics oligosaccharides

Prebiotics oligosaccharides (see table 1) have been defined as carbohydrates with a degree of polymerization (DP) from 2 to 10. However, oligosaccharides have recently been variously defined as a DP ranging from 2 to 20 or more. Recently, the International Union of Biochemistry and International Union of Pure and Applied Chemistry (IUB-IUPAC) Joint Commission on Biochemical Nomenclature stated that the borderline between oligo- and polysaccharides can not be drawn so strictly. However, the term oligosaccharide is commonly used to refer to defined structures as opposed to a polymer of unspecified length. The same approach is used for oligosaccharides of non human-milk origin as long as they have defined structures (Chapman and Hall, 1990).

Free oligosaccharides are natural constituents of all mammal milks. In comparison to human milk, the concentrations of oligosaccharides in these milks are much lower, and their structure is less complex as mentioned by Boehm and Stahl (2004, 2007).

Main oligosaccharides are:

Galacto-oligosaccharides

Fructo-oligosaccharides

Isomalto-oligosaccharides

Xylo-oligosaccharides

Human milk –oligosaccharides

Table 1 Some candidate prebiotic compounds**.**

Prebiotic	Production method	Reference
Inulin [Fructooligosaccharide (FOS)]	Hot water extraction from chicory root (followed by enzymatic hydrolysis) or polymerization of fructose monomers	Bornet *et al.*, 2002
Galactooligosaccharide (GOS)	Enzymatic lactose transgalactosylation	Teuri and Korpel, 1998
Xylooligosaccharide (XOS)	Enzymatic hydrolysis of plant xylans	Imaizumi *et al.*, 1991
Isomaltooligosacchairde (IMO)	Transglucosylation of liquefied starch	Morgan *et al.*, 1992
Lactulose	Isomerization of lactose	Salminen and Salminen, 1997

2.3.1.1. Galacto-oligosaccharides

Galacto-oligosaccharides (GOS) are principally formed by enzymic treatment of lactose by β-galactosidase to produce several oligomers of different chain lengths (Prenosil, *et al*., 1987). Galacto-oligosaccharides can be produced from lactose in cow's milk, but the main raw material for its production for commercial products is usually whey-derived lactose (Yanahira, *et al*., 1995). Further more GOS are stable at high temperatures in acidic conditions and the calorific value of these oligosaccharides is only 1.7 kcal/g. Which makes them of particular interest to the food and drink industry, for both their prebiotic properties, and their use as sweeteners, especially in confectionary, acidic beverages and fermented milks (Watanuki, *et al*., 1996)? Galacto-oligosaccharides are nondigestible, carbohydrate-based food ingredients that can enhance health related physiological activities (production of short chain fatty acids (SCFA), energy transduction in colonocytes, growth, and cellular differentiation of colonic epithelial cells, lipid, and carbohydrate metabolism), which can expand protection from infection; decrease the number of potentially pathogenic bacteria; facilitate the normal functions of the gut; stimulate the absorption of some minerals and decrease blood lipids content (Broek, *et al*., 2008). Prebiotic selectively increase the beneficial microbiota of the intestine, leading to health benefits that are extensively recognized by Macfarlane, *et al*., (2008). Because of their stability, GOS can be integrated into a wide variety of foods, where they have a pleasant taste, and can increase the texture and mouthfeel of foods, as well as acting as bulking agents. Because of this, GOS and fructo-oligosaccharides (FOS) are presently used in a wide range of commercial goods, including infant formulas, dairy products, soups, sauces, breakfast cereals, beverages, snack bars, ice creams, bakery products, animal feeds, and as sugar replacements (Yang and Silva, 1995).

2.3.1.2. Fructo-oligosaccharides

Fructo-oligosaccharides (FOS) are non-traditional sugars that can not be hydrolyzed by gastrointestinal enzymes. They have a low caloric value and can raise beneficial effects to the host via the selective stimulation of indigenous bacteria like bifidobacteria and lactobacilli (Mussatto and Mancilha, 2007; Teitelbaum and Walker, 2002).

A. Description and structure

Fructo-oligosaccharides among the group of oligosaccharides and are isolated from plants. They consist of three to ten monosaccharide units joined by α-glycosidic bonds (1-2) between terminal fructose and glucose (Tamine, *et al*., 1995). Perrin, *et al*., (2002) reported that the term FOS indicates to the inulin-type fructans. In the natural sources of FOS, the

molecule size is widespread (DP ranging from 2 to 60). Because the biological activity of prebiotics depends on the molecular size, it is mostly important to consider the molecular size allocation for reviewing clinical data on fructans. Long chain FOS are prepared from inulin from which the short chain FOS (DP 2–6) have been largely removed and then contain predominantly large molecules with a DP between 7 and 60. Roberfroid (2007) reported that FOS is produced by a totally different method. Using the fungal enzyme beta-fructosidase, derived from *Aspergillus niger*, FOS is enzymatically synthesized using a process called transfructosylation. Flamm, *et al*., (2001) have estimated the caloric value of FOS and found that the energy yield for the host would be in the range of 1.5 kcal/g to 2.0 kcal/g. Roberfroid, (1993) reborted that, by using method founded on lipogenesis balance stated that the caloric value of FOS from 1.0 to 1.5 kcal/g.

Fructo-oligosaccharides are ready in some foods such as chicory, yacon, artichoke, garlic onion, tomato, wheat, asparagus, leek, honey, rye, brown sugar, barley, triticale, beer, lettuce, burdock, beet root, apples, bulbs like red lilies, and oats (Table 2).

Source	Scientific name	Fructose units	Fructooligosaccharides (%) in fresh material
Banana	*Musa* spp.	2	0.3–0.7
Rye	*Secale cereale*	2	0.5–1.0
Leek	*Allium ampeloprasmus*	*n*[a]	2.0–10.0
Wheat	*Triticum asetivum*	*n*	0.8–4.0
Garlic	*Allium sativum*	*n*	1.0–16.0
Chicory roots	*Cichorium intybus*	*n*	15.0–24.0
Asparagus shoot	*Asparagus officinalis*	2–4	2.0–3.0
Jerusalem artichoke	*Heliantus tuberosus*	2	16.0–22.0
Globe artichoke	*Cynara scolymus*	2	3.0–10.0
Onions	*Allium cepa*	2–4	1.1–7.5
Salisfy	*Scorzonera hispanica*	*n*	4.0–11.0
Dandelion	*Taraxacum officinale*	*n*	12.0–15.0
Dahlia	—	*n*	13.0
Burdock	—	2–4	3.6

[a]*n* is either >4 or number of individual fructose units not described as yet.

Table 2 Natural occurrence of fructooligosaccharides was described by Mitsuoka, *et al.*, (1987); Roberfroid, *et al.*, (1993) and Modler (1994).

B. The effects

Gibson and Wang, (1994); Roberfroid, *et al*., (1998) reported that FOS and inulin have bifidogenic impact on host when consumed at a dose of 5g/day for oligofructose and ≤ 8

g/day for inulin, they importantly modify the composition of the intestinal (faecal) flora, selectively increasing the numbers of Bifidobacteria and reducing the deleterious bacteria.

2.3.1.3. Isomalto-oligosaccharides

Isomalto-oligosaccharides (IMO) are produced from glucose by enzymatic transgalactosylation (Hayashi, *et al*., 1994; Vetere, *et al*., 2000). It is a sugar replaces with 40% of the sweetness of sucrose and has been used widely in different foods and drinks (Kaneko, *et al*., 1995). Isomalto-oligosaccharides have been used as a sweetener in Japan for years. It is made from starch and consists mainly of oligomers with two to four degrees of polymerization, such as isomaltose, panose and isomaltotriose; these oligomers contain α 1 →6 glucosidic linkage (Kohmoto, *et al*., 1991). They resist endogenous digestion was record by Kohmoto, *et al*., (1992).

A. Description and structure

Isomalto-oligosaccharides are found naturally in different fermented foods such as sake, miso, or soy sauce but also in honey (Playne and Crittenden, 2004). The IMO means glucosyl saccharides with only α-(1→6) linkages; commercial IMO syrup is generally accepted as a mixture of glucosyl saccharides with both α-(1→6) linkages and α-(1→4) linkages (Yun, *et al*., 1994). Moreover, branched IMOs produced with dextransucrase, known as glucooligosaccharides (GOSs) (Paul, *et al*., 1992; Remaud-Simeon, *et al*., 1994), oligodextran created by controlled-hydrolysis of dextran (Mountzouris, *et al*., 2002), and non-reducing IMO-alditols produced through dextransucrasecatalyzed glucosylation of alditols such as mannitol, glucitol, maltitol, (Demuth, *et al*., 2002) are also assumed as IMOs. Branched IMOs (GOS) produced from saccharose and maltose by *Leuconostoc mesenteroide* enzymes were tested in vitro by substrate utilization tests with sundry human gut bacteria (Djouzi, *et al*., 1995; Wichienchot, *et al*., 2003) and *in vivo* in gnotobiotic rats inoculated with human fecal flora (Djouzi and Andrieux, 1997).

B. The effects

Isomalto-oligosaccharides have obtained interest as food additives because they can replace partially or totally, liquid sugar syrups, giving new functionalities to the product. Indeed, IMO are about half as sweet as saccharose and therefore can be used to produce different sweetness profiles. They can also be added to beer as non-fermentable sugar syrups to adjust sweetness and mouthfeel. They have been identified as good humectants with low viscosity and water activity but highmoisture retaining capacity (Takaku, *et al*., 1988). Thus are, they, able to maintain texture, prohibit microbial damage, and retard degradation in food

(Yoo, *et al.*, 1995). A recent study on the quality characteristics of sponge cake formulated using, in various proportions, IMOs as a sweetener to replace saccharose, which gave positive microbiological, physicochemical, and sensory evaluations (Ching-Ching, *et al.*, 2008).

2.3.1.4. Xylo-oligosaccharides

Xylo-oligosaccharides (XOS) are naturally available in bamboo shoots, which are also produced from xylan, a major component of hemicelluloses (Vazquez, *et al.*, 2000). Xylo-oligosaccharides are made up of xylose units and can be produced by enzymatic hydrolysis from xylan, which is the major component of plant hemicelluloses and therefore readily available in nature (Domínguez, *et al.*, 2003). Xylo-oligosaccharides are recorded to be preferentially fermented by bifidobacteria in vitro. Pure culture studies have indicated that XOS are metabolised by many bifidobacteria *B. bifidum, B. longum, B. catenulatum, B. lactis and B. adolescenti*s (Crittenden, *et al.*, 2002).

A. Description and structure

The structures of XOS differ in degree of polymerization (DP), monomeric units, and types of linkages. Generally, XOS are mixtures of oligosaccharides formed by xylose residues linked through β-(1→4)-linkages (Aachary and Prapulla, 2008). The number of xylose residues implicated in their formation can vary from 2 to 10 and they are known as xylotriose, xylobiose, and etc. For food applications, xylobiose (DP = 2) is considered to be a xylooligosaccharide (Vazquez, *et al.*, 2000).

Production of XOS can be achived by chemical methods, direct enzymatic hydrolysis of a susceptible substrate (Katapodis, *et al.*, 2002), (Katapodis and Christakopoulos, 2005) or combination of enzymatic and chemical treatments (Kokubo and Ikemizu, 2004); (Yang, *et al.*, 2005).

B. The effects

Xylo-oligosaccharides get better food quality, providing a change in physico-chemical characteristics, flavor and stimulating the activity of Bifidobacterium in the intestinal tract (Nakano, *et al.*, 1998). The use of XOS as an ingredient in food products is due to their stability towards a wide range of pH (2.5 to 8.0) and temperature, the selective metabolism by bifidobacteria, the increased production of volatile fatty acids, the reduction of stomach ulcer lesions (Parajo, *et al.*, 2004) and the acceptable odor (Hsu, *et al* ., 2004). Fooks and Gibson, (2002) reported that, mixtures of inulin: FOS and FOS: XOS were effective in preventing growth of *E. coli* and *Salmonella enteritidis.*

The antimicrobial potential displayed by each of the probiotics used appeared to be based on the carbohydrate source. In poultry, XOS decreased ileal lactic acid concentration, and increased cecal butyric acid and total volatile fatty acid concentrations. Xylo-oligosaccharides were quickly fermented in the cecum, but had little influence on the overall bacterial community profile (Graham, *et al*., 2004). Xylo-oligosaccharides (alone or as active components of pharmaceutical preparations) display a range of biological activities vary from the prebiotic effects related to gut modulation. The other effects for XOS include antioxidant activity (conferred by phenolic substituents), blood- and skin-related effects, antiallergy, anti-infection and anti inflammatory properties, immunomodulatory action, anti-hyperlipidemic effects.

2.3.2. Health benefits of prebiotics

The plurality of the effects demanded by the prebiotics are related with optimized colonic metabolism and function, such as an increase in the expression or change in the composition of short chain fatty acids, increased fecal weight, a reduction in luminal colon pH, a decrease in nitrogenous end products and reductive enzymes, an increased expression of the binding proteins or on definite biomarkers in the field of lipid and mineral metabolism and immune system modulation (Bournet, *et al.,* 2002; Forchielli and Walker, 2005).

a) Effects on combinations of microbiota

Prebiotics like FOS, trans-galactooligosaccharides (TGOS) and Inulin as well as their synbiotic combination with probiotic bacteria (strains of *L. plantarum*, *L. paracasei*, or *B. bifidum*) increased bifidobacteria and lactobacilli or inhibited different human- and animal pathogenic bacterial strains (*Clostridium* sp., *E. coli*, *Campylobacter jejuni*, *Enterobacterium* sp., *Salmonella enteritidis,* or *S. typhimurium*) in vitro in mice (Asahara, *et al*., 2001), piglets (Bomba, *et al.,* 2002), or humans (Langlands, *et al.,* 2004). Furthermore, a combination of prebiotics like polydextrose and lactitol influences the microbial ecosystem of the gastrointestinal tract of rat and promote the immune response by increasing the secretion of immunoglobulin.

b) Immuno-modulatory effects

The functional foods are recorded to promote the immunity of the consumers. In fact, the dietary ingredients and their fermentation metabolites are in close contact with the gutassociated lymphoid tissue (GALT) which is the part of the huge intestinal immune system. The presence of food in the small intestine may be important for adequate function and development of GALT (Scheppach, *et al.,* 1992). Truly, Palma, *et al.,* (2006) have

described that *β*-glucuse stimulate innate immune reactions by binding to selective receptors (such as dectin-1) mainly expressed on M2 macrophages.

c) Effects on prevention of cancer

Fermentation of prebiotics led to the production of short-chain fatty acids (SCFA) which expand many effects on colonic mucosa. Butyric acid is used by the epithelial cells of the colon mucosa as energy source, being in addition a growth factor (Bugaut and Bentéjac, 1993).

Recent preclinical studies have recorded that butyrate would be chemopreventive in carcinogenesis (Scheppach and Weiler, 2004) or protector agent against colon cancer by enhancing cell differentiation (Kim, *et al.,* 1982). In vitro study on human colonic lines L97 and HT29 (representing early and late stages of colon cancer), fermentation supernatant fractions of inulin showed a significant growth-inhibiting and apoptosis inducing effects in the human colon tumour cells.

d) Effects on lipid metabolism

Prebiotic has also been demonstrated to exert an effect on hepatic lipid metabolism. Inulin and oligofructan have shown a physiological effect on cholesterol and triglyceride levels in rats by decreasing postprandial cholesterolemia and triglyceridemia by 15% and 50% respectively (Delzenne, *et al.,* 2002 and Fiordaliso, *et al.,* 1995).

Recently, combination of high protein diet (HP) with a high fibre diet (HF) resulted in an increased anorexigenic and insulinotropic hormone, glucagon-like peptide-1 (GLP-1), and an progress on glucose tolerance or lipid profiles in HF diet and the diets containing inulin delayed the lowest plasma triglyceride and total cholesterol levels (Reimer and Russell, 2008)

e) Effects on minerals absorption

Effects of dietary factors on calcium absorption may be modulated by genetic factors, including specific vitamin D receptor gene polymorphisms (Abrams, *et al.,* 2005). Furthermore, studies in animal models have shown increased calcium availability with inulin and oligofructose in the diet (Scholz-Ahrens and Schrezenmeir, 2007).

Additionally, *Lactobacillus* and *Bifidobacterium* of populations were significantly increased in the caecal content microflora (Tako, *et al.,* 2008). In rat model, both native Inulin and reformulated Inulin exerted similar effects as to caecal fermentation by production of

short-chain fatty acids, especially butyric acid and stimulation of Ca and P digestive absorption and affects the bone mineral density (Demigné, *et al.*, 2008).

2.4. Human milk oligosaccharides

2.4.1. Description and structure

Human milk oligosaccharides (HMOs) are complex glycans that are present at very high concentrations in human milk but not in infant formula (Bode, 2009). The amount of HMOs are differ depending on individuals and the lactation periods, while, it can reach up to 15 g/l which is equal to, or more than, the amount of proteins in human milk (Coppa, *et al.*, 1993; Kuntz, *et al.*, 2008). Human milk is a complex biological fluid consisting proteins, lipids, vitamins, carbohydrates, and minerals. Breast-fed infants mostly have promoted resistance to infectious diseases and better cognitive functions (Lawrence and Pane, 2007; Smith *et al.*, 2003). Erney *et al.*, (2000) recorded that oligosaccharides are the third largest solid constituent of human milk after lactose and lipid.

Monomers of human milk oligosaccharides

Kunz and Rudloff, (2006); Bode, (2009) mentioned that HMOs are comprised by the five monosaccharides (Fig. 1.): D-glucose (Glc), D-galactose (Gal), N-acetylglucosamine (GlcNAc), L-fucose (Fuc), and sialic acid (Sia; N-acetyl neuraminic acid [Neu5Ac]).

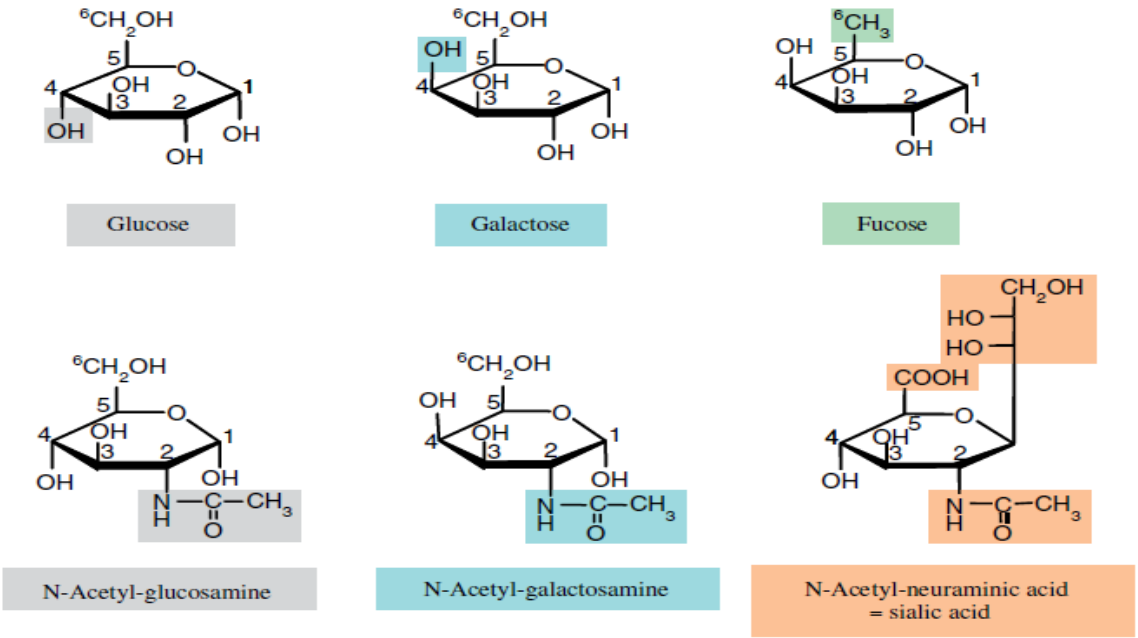


Fig. 1 Components of oligosaccharides of human milk, drawing by Kunz and Rudloff, (2006).

The structures of HMOs (Fig. 2.) are very diverse and complicated. Having different compositions and glycosyl linkages, more than 200 isomers were found with various degrees of polymerization (DP 3 to 20). Regardless of their structural complexity, HMOs share some popular backbones. Most of HMOs have the lactose (Galβ1-4Glc) residue at the reducing end.

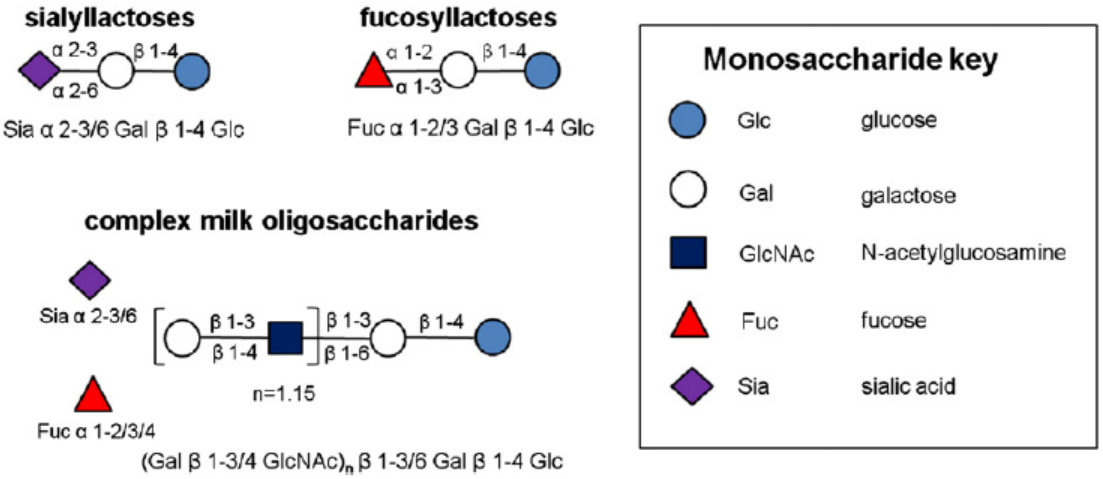


Fig. 2 Structural composition of milk oligosaccharides. (Bode, 2009).

Bode, (2009) reported that Gal in lactose can be sialylated in α-(2, 3) and/or α-(2, 6) linkages to form 3′-sialyllactose and 6′-sialyllactose, respectively. Lactose can also be fucosylated in α-(1, 2) and α-(1, 3) linkages to form 2′-fucosyllactose and 3′-fucosyllactose, respectively. These trisaccharides are called the short-chain milk oligosaccharides. To form the complex milk oligosaccharides, N-acetyllactosamine (Galβ1-3/4GlcNAc), lactose or polylactosamine backbone can be sialylated in α-(2, 3) and/or α-(2, 6) linkages and/or fucosylated in α-(1, 2), α-(1, 3), and/or α-(1, 4) linkages. Approximately 200 different complex oligosaccharides have been identified in human milk.

2.4.2. Functions of human milk oligosaccharides

A hundred years ago advantage of milk oligosaccharides started after observing that the carbohydrate fraction is most likely responsible for the development of a bifidogenic flora in breastfed children (Kunz *et al.,* 2000). Nowadays, milk oligosaccharides are supposed to be useful for the human milk fed infant with consider to their prebiotic and anti-infective properties.

Human milk oligosaccharides have certain biological functions. Like functions may include prebiotic activity, anti-adhesive activity, anti-inflammatory properties, modification of the entire complement of cell surface sugars, a role in brain development, influencing growth-

associated with characteristics of intestinal cells and absorption of minerals (Bode, 2006; Hickey, 2009; Kunz and Rudloff, 2006 and Newburg, *et al*., 2005). But, there are very few commercial products on the market that capitalise on these functions. This is fundamentally in order to the truth that the large quantities of human milk oligosaccharides needed for clinical trials are unavailable. In compare, commercial oligosaccharides such as galacto-oligosaccharides and fructooligosaccharides are present in specific products such as infant formula, which are actually marketed based on prebiotic health claims (Fanaro, *et al*., 2005). Anyway, the structure and composition of commercial oligosaccharides are very different from the structure and composition of human milk oligosaccharides.

For example specific biological properties, such as prohibition of pathogen adhesion, seem ascribed mostly to human milk oligosaccharides given that a single group of oligosaccharides (galacto-oligosaccharides or other) invariably can not match the anti-adhesive properties of the highly diverse human milk oligosaccharides structures. Indeed, human milk oligosaccharides structurally mimic epithelial cell surface carbohydrates and thus function as decoys to which pathogens can bind instead of the host, thereby prohibiting infection (Kunz, *et al*., 2000).

2.4.2.1. Prebiotic function

The prebiotic effect of human milk has been studied from the middle of the 20th century. György *et al.* (1954) mentioned that the components of human milk have been known to enhance the growth of *Bifidobacterium bifidum* by their prebiotic effect. Recent studies showed that this prebiotic effect (also known as Bifidogenic effect) is connected to the oligosaccharide in human milk. It was recorded that the infant-borne bifidobacteria preferentially consume small mass HMOs initially then consume completely in a late stage of cell growth (LoCascio *et al.*, 2007).

Functional oligosaccharides are substrates that can only be consumed by a limited number of bacteria, stimulating thus their growth. Within the group of bacteria present in the gastrointestinal tract, the bifidobacteria and lactobacilli are those that most utilize oligosaccharides being considered as the only microorganisms able to beneficially affect the host's health (Mikkelsen and Jensen, 2004; Vernazza, *et al*., 2005). Human milk acts as an effective prebiotic (ie, a food that selectively stimulates the growth of beneficial bacteria in the colon). The high concentrations of lactose and nondigestible oligosaccharides found in human milk enhance the colony formation of *Bifidobacteria* spp. and *Lactobacillus* spp. (Yoshioka, *et al*., 1983).

Prebiotic effects of HMOs, this highly simplified scheme shows that desired (light) and undesired (dark) bacteria have various capabilities of metabolizing HMOs. In the presence of HMOs (right), the desired bacteria metabolize HMOs and thrive while undesired bacteria cannot metabolize HMOs (Fig. 3.). Metabolites from bacterial HMOs degradation, e.g., short-chain fatty acids, create an environment that also benefits the growth of desired bacteria. In the absence of HMOs (left), both desired and undesired bacteria can grow.

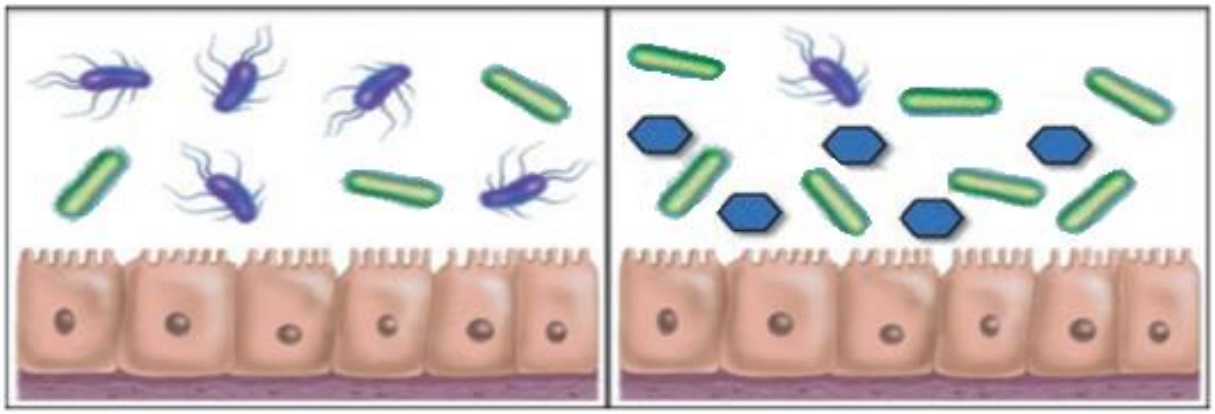


Fig. 3 Some pathogens need to attach to the intestinal epithelial cell surface prior to invading the host, Modefied from (Bode, 2009).

Breast fed infants are clearly different from those of formula-fed infants and are characterized by high lactate, low pH, and high acetate (Ogawa, *et al.*, 1992). Intestinal colonization with *Bifidobacterium* spp. and *Lactobacillus* spp. prevents the growth of *Clostridium* spp. and other pathogenic organisms (Ogawa, *et al.*, 1992; Lundequist, *et al.*, 1985) and has been associated with a decrease in severity of gastroenteritis. Intestinal colonization with *Bifidobacterium* spp. and *Lactobacillus* spp. is suspected to have gut-barrier functions, to give maturation signals for the gut-associated lymphoid tissues, and to balance the generation of pro- and anti-inflammatory cytokines, thereby creating healthy interactions between the host and microbes that are required to help regulate inflammatory responses in the developing infant gut (Schiffrin and Blum, 2002).

2.4.2.2. Function against pathogens (prevence of adhesion)

A critical pathogens factor for many infectious diseases such as diarrhea for example is the ability of microbial pathogens to adhere to the mucosal surface and their subsequent spreading, colonization and invasion (e.g., for *Escherichia coli*, *Helicobacter jejuni*, *Shigella* strains, *Vibrio cholerae* and *Salmonella* species) in the gut (Beachey, 1981; Ofek and Sharon, 1990). Bacterial adhesion is oftentimes a receptor-mediated interaction between structures on

the bacterial surface and complementary ligands on the mucosal surface of the host (Karlsson, 1995). Intestinal colonization with *Bifidobacterium* spp. and *Lactobacillus* spp prevents the growth of *Clostridium* spp. and other pathogenic organisms (Ogawa, *et al*., 1992; Lundequist *et al*., 1985) and has been associated with a decrease in severity of gastroenteritis.

Human milk oligosaccharides components actively protect the infant from pathogenic infection and facilitate the basing of the microbiota, the latter of which is needed to activate the mucosal immune system. Such as, human milk (HM) constitutes a "communication vehicle" between the mother and the infant that minimizes the infant's disease risk, (Forchielli and Walker, 2005, Brandtzaeg 2003 and Walker, 2004). Additionally, there is compelling evidence that breastfeeding confers longer-term risk reduction for autoimmune diseases such as celiac disease (Greco, *et al*., 1998). In the short term, epidemiological and clinical data supply strong evidence that HM feeding reduces the incidence, severity, or both of infectious diseases (Heinig, 2001).

There are two possibilities supposed for potential inhibitors of pathogen adhesion (Fig. 4.)

(1) HMOs are soluble receptor analogues of epithelial cell-surface carbohydrates, and vie with epithelial ligands for pathogens by binding to proteins on the pathogens (lectins or haemmaglutinnins);

(2) HMOs may also adjust gene expression associated with enzymes change the cell-surface glycome which could interfere to adhesion, colonization and proliferation of pathogens (Kunz and Rudloff, 1993; Bode, 2009)

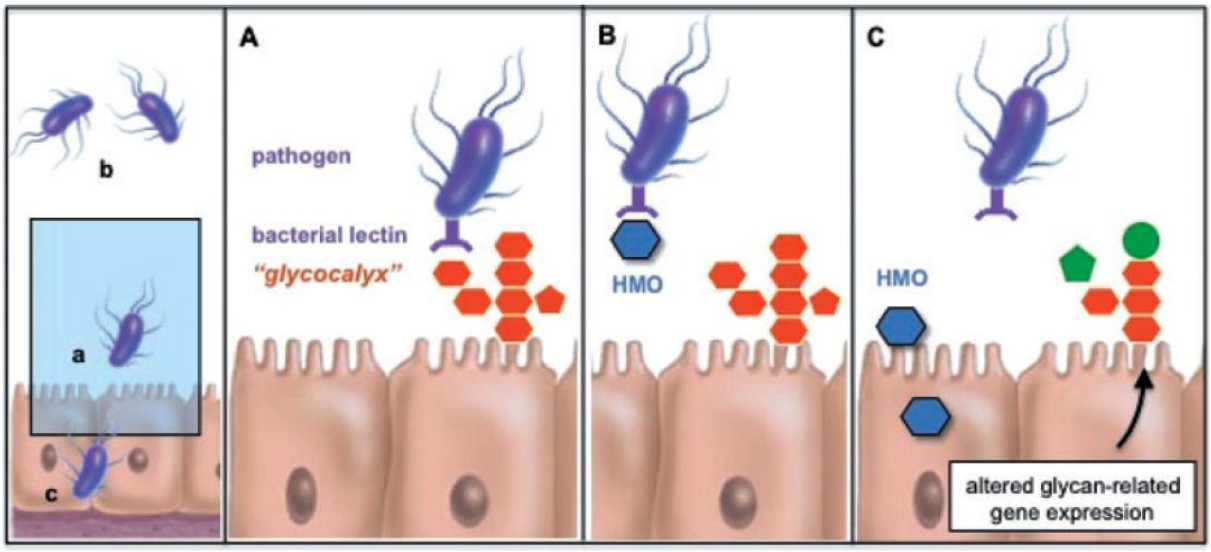


Fig. 4 Most bacteria (commensals and pathogens) express glycan-binding proteins (lectins), that bind to glycans on the host's epithelial cell surface (A), which is essential for bacteria to attach (a), and to proliferate and colonize the intestine (b). Some pathogens need to attach to the

intestinal epithelial cell surface prior to invading the host (c). HMOs are structurally similar to the intestinal epithelial cell surface glycans. They can serve as bacterial lectin ligand analogs and block bacterial attachment (B). Human milk oligosaccharides (HMOs) may also alter the intestinal epithelial glycosylation machinery and modify the cell-surface glycome ("glycocalyx"), which could impact bacterial attachment, proliferation, and colonization (C) (Bode, 2009).

2.4.2.3. Development of centeral nervous system

Sialic acid (Fig. 5.) is a part of human milk mligosaccharides (Kunz and Rudloff, 2006).

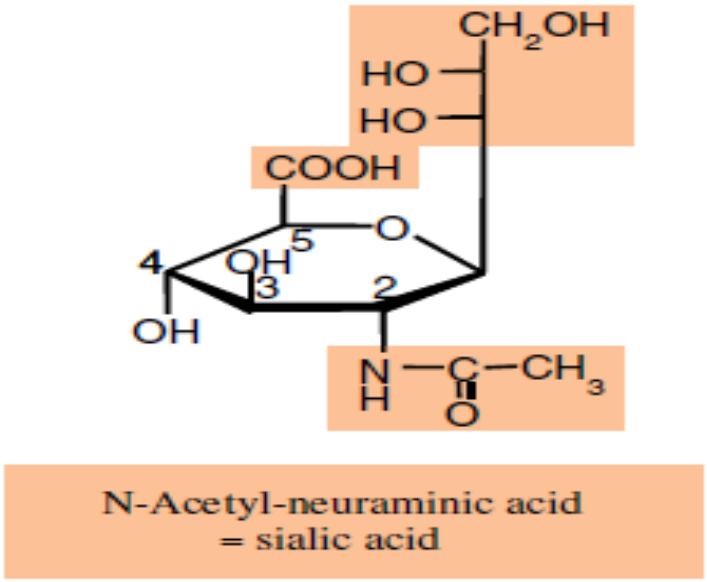


Fig. 5 Sialic acid structure.

Early human milk is a rich source of sialic acid, N-acetylneuraminic (Carlson, 1985). More studies showed that children who were breast-fed as babies reach higher scores on intelligence tests than those who were bottle-fed (Rodgers, 1978; Fergusson *et al*, 1982; Lucas *et al*, 1992, 1998). On rate, scores are 2–9 points higher, a difference that is considered biologically significant. The difference becomes more pronounced as the period of breastfeeding increases (Dewey *et al*, 1995). Morgan and Winick (1980) reported that exogenous sialic acid administered by intraperitoneal injection increased the production of ganglioside sialic acid in the brain and improved learning ability in well-nourished and malnourished rat pups.

In a retrospective study, Menkes (1977) obtained a significantly greater incidence of bottle feeding within learning-disabled children than among controls being followed for other neurological symptoms. Rodgers (1978) showed a large, stratified sample of British children covariates included social class, parental interest in education, material home conditions,

parental education, family size and birth rank, and age at weaning. After control of confounding variables, there was a significant advantage to breast-fed children on a picture vocabulary test at 8 years of age and on mathematics, nonverbal ability, and sentence completion at 15 years. Recently, Mortensen *et al*., (2002) also reported that period of breastfeeding was associated with significantly higher scores on all components of the Wechsler adult intelligence scale. Rodgers (1978) suggested possible mediating factors might be differences between breast and bottle milk osmotic load or protein and lipid concentrations or differences in the feeding situation such as infection risk and psychological effects.

2.4.2.4. Absorption of minerals

Milk of many species contains high concentrations of phosphorus and calcium (Holt *et al*., 1981). Caseins and minerals in milk are in dynamic equilibrium between the soluble and micellar phases, and the partitioning depends upon minerals, temperature, and the pH value. When milk pH reduces from 6.7 to 6.0, soluble calcium raises by 20% and soluble phosphorus by 15% (Ezeh and Lewis, 2011).

Phosphate and calcium can form many various types of complexes, like dicalcium phosphate, micellar calcium phosphate, dicalcium phosphate dihydrate, octacalcium phosphate, β-tricalcium phosphate, hydroxyapatite, amorphous calcium phosphate, tricalcium citrate dihydrate and dimagnesium phosphate (Gaucheron, 2005). Adequite calcium supply is an important prerequisite for normal bone mineralization and subsequently for normal growth and development of preterm infants. It is commonly accepted that the efficiency of calcium absorption from human milk is significantly higher than that from a preterm formula.

There are many factors in human milk that might influence the calcium absorption. Among others, like peptides or lipids, oligosaccharides could also contribute to the high efficiency of calcium absorption from human milk (Lönnerdal, 1997).

Boehm *et al*., (2002) reported that in adults, it can be shown that dietary oligosaccharides enhance calcium absorption, the mechanism by which oligosaccharides promote calcium absorption is not well understood but probably this effect has been related to the bifidobacteria-stimulating capacity of the prebiotic substrate.

2.4.3. Utilization of HMOs by biffidobacteria

Human milk oligosaccharides (HMOs) are minimally digested by the infant and persist to positive and negative gut microbiota. The dominant component of the intestinal microflora for healthy infants, which were born normally and fully breastfed, are bifidobacteria.

Bifidobacteria belong to the phylum *Actinobacteria* which encompass Gram-positive bacteria characterized by chromosomes enriched for cytosine and guanine content (Ventura, *et al*., 2007). Bifidobacteria show remarkable adaptations to use and metabolize complex oligosaccharides as a carbon and energy source (Lee and O'Sullivan, 2010). In breast-fed infants, the basic carbon sources available for the developing intestinal microbiota are human milk oligosaccharides (HMOs; (Kunz, *et al*., 2000)) and specific bifidobacteria can gain access to N- and O-glycans in mucins or milk proteins (Garrido, *et al*., 2012b; Ruas-Madiedo, *et al.*, 2008). Only a few bacterial species have been shown to use these substrates (Marcobal, *et al*., 2010), and the molecular mechanisms involved in HMOs utilization in bifidobacteria are beginning to be understood (Garrido, *et al.*, 2012a). In adults, diet delivers the intestinal microbiota a huge variety of oligo- and polysaccharides, which are resistant to enzymatic degradation in the intestinal lumen and also reaches distal portions of the intestine. Different bifidobacterial species are capable of metabolizing complex oligosaccharides usually from plant origin such as amyloses and cellodextrins (Pokusaeva, *et al.*, 2011), raffinose (Dinoto, *et al.*, 2006), arabinooligosaccharides (Lagaert, *et al*., 2010; Van Laere, *et al*.,1997), xylooligosaccharides (Gilad, *et al*., 2010), fructooligosaccharides and inulin (Omori, *et al*., 2010; Perrin, *et al*., 2001; Rossi, *et al*., 2005), galactans and galactooligosaccharides (GOS; (Barboza, *et al*., 2009; Goulas, *et al*., 2009; Hinz, *et al*., 2005; O'Connell Motherway, *et al*., 2011)) among several others.

Bifidobacteria grew on caw milk (CM), lactose, HM and on HMOs. Bifidobacterial strains were resistant to lysozyme (Rocková, *et al.*, 2011). Bioinformatic analysis revealed several physiological traits that could partially explain the successful adaptation of this bacterium to the colon, also have been isolated from infant and adult human faeces, from faeces of suckling calf, from human vagina and from sewage (Reuter, 1963).

Genomes of *B. longum* subsp. *infantis* encode a suite of expected intracellular glycosidases lacking identifiable transmembrane domains, secretion signals or Gram-positive cell wall anchors in addition to a multitude of transporters, encouraged the hypothesis that this bacterium imports intact oligosaccharides as the rate determining step in HMOs metabolism (Sela and Mills, 2010). This is in agreement with the *B. longum* subsp. *infantis* HMOs utilization glycoprofile that indicates higher molecular weight HMOs are not metabolized, evocative of a translocation barrier. Extracellular hydrolysis would not display this glycoprofile due to structural redundancy in serially integrated HMOs subunits (LoCascio, *et al*., 2009). Genomic analysis suggests that *B. longum* subsp. *infantis* evolved from a plant derived glycan utilization genotype, to be competitive in the infant colon. Interestingly, all

available carbon sources in this environment are oligosaccharides from human origin, including a significant concentration of HMOs arriving undigested to the distal colon, like intestinal secretions and glycoconjugates from epithelial cells. The *B. longum* subsp. *infantis* genome encodes several gene clusters active on HMOs or derivatives including sialidases and fucosidases. These glycoside hydrolases cleave substituted termini to expose HMOs core structures such as lacto-N-tetraose (LNT; Galß1–3GlcNAc ß1–3 Galß1–4Glc). HMOs-related gene clusters are distributed throughout the *B. longum* subsp. *infantis* chromosome and are clearly absent from genomes of the phylogentically-near subspecies longum (Sela, *et al*., 2008).

2.5. Composition of mammalian milk

Milk production is a necessary part of the national economy in several countries, especially in the Mediterranean and Middle East regions (FAO, 2003). Milk composition differs according to several factors, such as animal, feed and environment. Milk from all mammals studied so far contains an oligosaccharide fraction. Human and elephant milks contain the greatest concentrations of oligosaccharides and these oligosaccharides have the greatest structural complexity (Kunz *et al.*, 1999). The physico-chemical characteristics of milk are related to its composition for a particular animal species. Compositions of goat, sheep, cow and human milks are different (see table 3.). Sheep milk contains higher levels of total solids and major nutrient than cow and goat milk. Lipids in sheep and goat milk have higher physical characteristics than in cow milk, but physico-chemical indices (i.e., saponification, Reichert Meissl and Polenske values) vary between different records. Micelle structures in sheepare smaller than it in cow and goat. In the ruminants 75% of protiens are casine type milk, but in human, pigs and horses there is more albumine than casine. Caprine casein micelles contain more calcium and inorganic phosphorus, are less solvated, less heat stable, and lose β- casein more readily than bovine casein micelles.

Composition	Goat	Sheep[a]	Cow	Human
Fat (%)	3.8	7.9	3.6	4.0
Solids-not-fat (%)	8.9	12.0	9.0	8.9
Lactose (%)	4.1	4.9	4.7	6.9
Protein (%)	3.4	6.2	3.2	1.2
Casein (%)	2.4	4.2	2.6	0.4
Albumin, globulin (%)	0.6	1.0	0.6	0.7
Non-protein N (%)	0.4	0.8	0.2	0.5
Ash (%)	0.8	0.9	0.7	0.3
Calories/100 ml	70	105	69	68
Oligosaccharides	0.005	0.005	0.005	1

Table 3 Composition of mammalian milk.

Renneting parameters in cheese making of sheep milk are influenced by physico-chemical properties, including pH, larger casein micelle, more calcium per casein weight, and other mineral contents in milk, which cause differences in coagulation rate, coagulation time, curd firmness and amount of rennet required. Renneting time for goat milk is shorter than for cow milk, and the weak consistency of the gel is beneficial for human digestion but raises its cheese yield (Park *et al*., 2007). Cow milk contains a low concentration of oligosaccharides with a smaller number of structures (Gopal and Gill, 2000; Urashima *et al*., 2001). Bioactive peptides may be obtained from goat or sheep milk proteins since their primary structures are close to those observed for bovine proteins. For instance, caprine α-lactorphin was obtained after pepsin hydrolysis of α- lactalbumin (Bordenave, 2000). Data summrized in table 4 concerning the main minerals are available for goat, sheep, and cow and human milks.

	Goat[a]	Sheep[b]	Cow[a]	Human[a]
Calcium (mg)	1260	1950-2000	1200	320
Phosphorus (mg)	970	1240-1580	920	150
Potassium (mg)	1900	1360-1400	1500	550
Sodium (mg)	380	440-580	450	200
Chloride (mg)	1600	1100-1120	1100	450
Magnesium (mg)	130	180-210	110	40
Ca/P (mg)	1.3	1.3-1.6	1.3	2.1
Zinc (µg)	3400	5200-7470	3800	3000
Iron (µg)	550	720-1222	460	600
Copper (µg)	300	400-680	220	360
Manganese (µg)	80	53-90	60	30
Iodine (µg)	80	104	70	80
Selenium (µg)	20	31	30	20

Table 4 Mineral composition of goat, sheep, cow and human milk (Raynal-Ljutovac *et al*., 2008).

[a] Data compilation from Guéguen (1997) (per l).

[b] Data compilation from Guéguen (1997), Haenlein and Wendorff (2006) (per kg) and Paccard and Lagriffoul (2006a,b) (per kg).

Sheep milk presents the highest dry matter. Goat milk is distinguished by its high chloride and potassium content. Repartition of phosphorus, calcium and magnesium between the soluble and colloidal phases of milk are similar for cow and goat milks; sheep milk, however, has far lower solubility (Holt and Jenness, 1984).

For milk vitamins content, the goat and sheep milk demonstrated the high content in B vitamins especially niacin for both milks (table 5.). Nevertheless, goat milk is poor in folic acid and vitamin E. Both goat and sheep milk are lacking β- carotene, which is entirely converted into retinol.

		Goat[a]	Sheep[b]	Cow[a]	Human[a]
Fat soluble vitamins A	Retinol (mg)	0.04	0.08	0.04	0.06
	Beta carotene (mg)	0.00		0.02	0.02
D (µg)		0.06	0.18	0.08	0.06
E	Tocopherol (mg)	0.04	0.11	0.11	0.23
Water soluble vitamins B1	Thiamin (mg)	0.05	0.08	0.04	0.02
B2	Riboflavin (mg)	0.14	0.35	0.17	0.03
B3	Niacin (PP) (mg)	0.20	0.42	0.09	0.16
B5	Pantothenic acid (mg)	0.31	0.41	0.34	0.18
B6	Pyridoxin (mg)	0.05	0.08	0.04	0.01
B8	Biotin (µg)	2.00	nd	2.00	0.70
B9	Folic acid(µg)	1.00	5.00	5.30	5.20
B12	Cobalamin (µg)	0.06	0.71	0.35	0.04
	Ascorbic acid (mg)	1.30	5.00	1.00	4.00

Table 5 Vitamin content of goat, sheep and cow raw whole milks (per 100 g) (Raynal-Ljutovac et al., 2008).

nd: not determined

[a] Data compilation according to Jaubert (1997)

[b] Data compilation according to Paccard and Lagriffoul (2006a, b)

3. THESIS OBJECTIVES

Hypothesis of this work is that human milk oligosaccharides will support the growth of bifidobacteria in (*in vitro*) conditions.

The experimental and scientific works have been devoted to achieve the following aims:

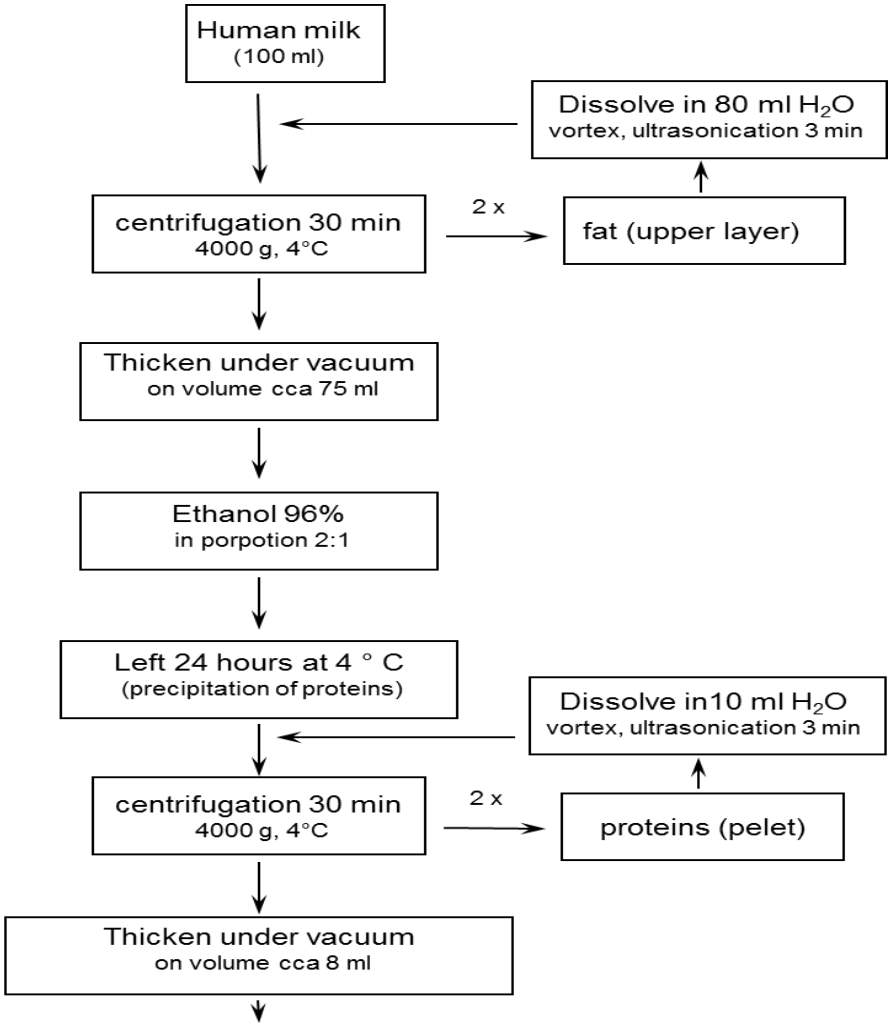
1) To isolate oligosaccharides; the samples were isolated from human, sheep and goat milk by using by gel-filtration chromatography (GLC) and screened the fractions by Thin-layer chromatography (TLC).

2) To test the ability of *B. longum* subsp. *longum* and *B. longum* subsp. *Infantis* to grow in human milk and to utilize human milk oligosaccharides. For this aim, five strains of bifidobacteria of human origin and 2 strains of bifidobacteria of animal origin were tested for growth in milk samples by using microtiter plate technique.

4. MATERIALS AND METHODS

4.1. Isolation of oligosaccharides from human milk

Oligosaccharides were isolated and purified according to the method by Ročková *et al*., (2011). At first, human milk was defatted by centrifugation at 4000 g for 30 minutes at 4 °C. After fat removal, pure ethanol (96%) was added then (in the ratio 2:1 v/v) and the mixture was kept 24 h at 4 °C. The centrifugation process was repeated again, and then the sample evaporated by vacuum evaporator (see Appendix, Fig. 1.) at 20 g at 40 °C. The residuum was dissolved in pure water (10 ml) and the precipitation process was repeated. For further removal of residual, protein was performed by precipitation with mixture of pure ethanol, dichlormethane (CH_2Cl_2) and pure water in the ratio 7:14:10 (v/v). After the removal of residual protein and dichlormethane, the extract was evaporated under vacuum (Fig. 6.).



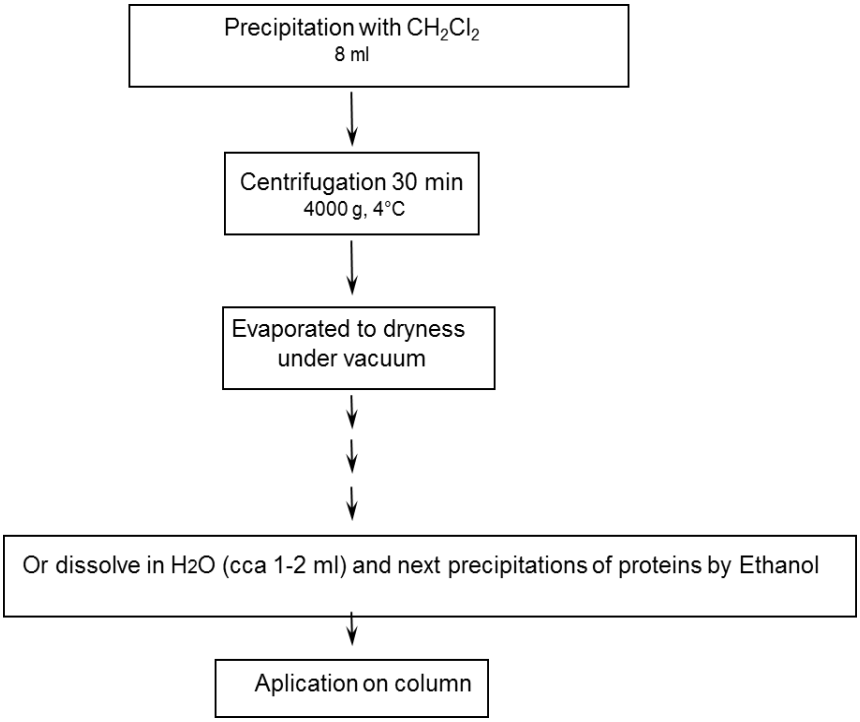


Fig. 6 Isolation steps of oligosaccharides from human milk.

The crude oligosaccharide extract was dissolved in water, and further purified by gel-filtration chromatography on a 1,6 cm x 180 cm column filled with Toyopearl HW- 40F (Tosoh Bioscience, GmbH) in 1% acetic acid as the mobile phase.

The principal of gel-filtration chromatography is the separation of sample compounds based on their different molecular weight. Separation is achieved by using a porous matrix to which the molecules, for steric reasons, have different degrees of access - i.e., smaller molecules have greater access and larger molecules are excluded from the matrix (Hagel, 2001). The process of gel filtration chromatography is shown on the picture below (Fig. 7.).

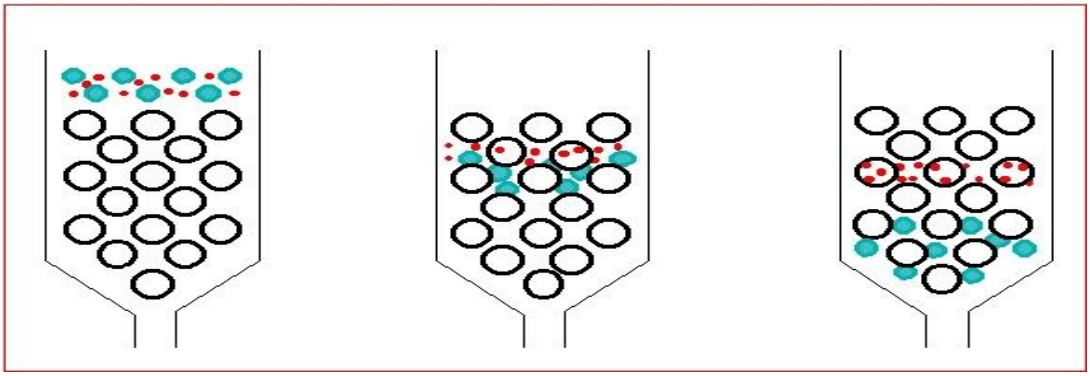


Fig. 7 Process of the Gel-filtration chromatography.

Fractions were collected by 50 drops into the tubes by Gilson FC 204 Fraction Collector (Gilson, Inc.). Each fraction has been screened by Thin-layer chromatography (TLC) before use with isopropanol-water- 25% ammonia solution (5:1:2, by vol.) as a mobile phase (visualisation by spraying with 10% sulphuric acid in ethanol and heating). Selected fractions containing oligosaccharides only mixed and lyophilized.

4.2. Growth of bacteria on human milk oligosaccharides

Bifidobacterial strains (Table 6.) were isolated and identified to the species level as described in Rada *et al.*, (2010).

Bifidobacterial strains	Origin
Bifidobacterium animalis subsp. *lactis 1*	Fermented milk product
Bifidobacterium animalis subsp. *lactis 2*	Fermented milk product
Bifidobacterium bifidum 1	Probiotic capsule
Bifidobacterium bifidum 2	Infant faeces
Bifidobacterium bifidum 3	Infant faeces
Bifidobacterium longum 1	Infant faeces
Bifidobacterium longum 2	Probiotic capsule

Table 6 Bacterial strains tested for utilization of oligosaccharides, Ročková *et al.* (2011).

HMOs were added (1% w/w) to the complex medium (contained per 1: tryptone, 10 g; peptone, 10 g; yeast extract, 5 g; sodium pyruvate, 1 g; tween 80, 1 ml; cysteine, 0.5 g) as the sole carbon source.

4.3. Microtiter plate technique

Microtiter plate (see Appendix Fig. 2.) is a flat plate with multiple "wells" used as small test tubes. A microplate typically has 6, 24, 96, 384 or even 1536 sample wells arranged in 2:3 rectangular matrixes. Each well of a microplate typically holds somewhere between tens of nanolitres to several millilitres of liquid. Wells can be either circular or square. To prepare the microtiter plate, microscope, micro tubes, petri dishes, incubator, micropipettes, anaerobic jar, syringes, centrifuge, agar plates, bifipufer, reflectoquant (Merck, Darmstadt, Germany), growth medium, dilution liquids and flame were used.

A suspension was prepared from a pure, 24 hours culture in the suspension medium. One and half ml of suspension medium (concentration 10^8/ml) were added to micro tubes then centrifuged for 4 minutes (16 000 g). The medium was discarded and the cells were washed by phosphate buffer (1.8 ml of phosphate buffer was taken by syringe; 0.3 ml for washing the wall of micro tubes and 1.5 ml mix with the cells). One ml of cells and phosphate buffer was added to dilution tubes and diluted to 10^{-5}. Ten µl of cells and phosphate buffer were transferred to the microtiter plate wells contained 90µl of human milk sample by using micropipettes. Then, microtiter plate was inserted into an anaerobic jar which provides anaerobic conditions for growth of bifidobacteria. Bifidobacteria were incubated with oligosaccharides dissolved in the complex medium for 24 hours in 37 °C in anaerobic jar (Anaerobic plus system, Oxoid). Numbers of colonies has been counted and lactic acid and pH were determined using Reflektoquant RQflex10 equipment (Merck, Darmstadt, Germany) with a Lactic Acid Test (Merck), (see Appendix Fig. 3.).

After that, 10µl of human milk which contain bifidobacterial cells was transferred from microtiter plate well to dilution tubes using micropipettes. Then diluted from 10^{-2} to 10^{-5}. Half ml of each dilute was transferred to petri dishes and then agar added. Petri dishes were inserted into an anerobic jar, and then anaerobic jar was kept in incubator at 37 °C for 48 hours. Colonies of bifidobaceria were counted after 48 hours.

5. RESULTS

5.1. Isolation of human milk oligosaccharides

Results of thin layer chromatography are showen in Fig. (8.), it's evident that fractions from 79 to 97 contain exclusively HMOs. These fractions are free of monosaccharides (lactose and glucose).

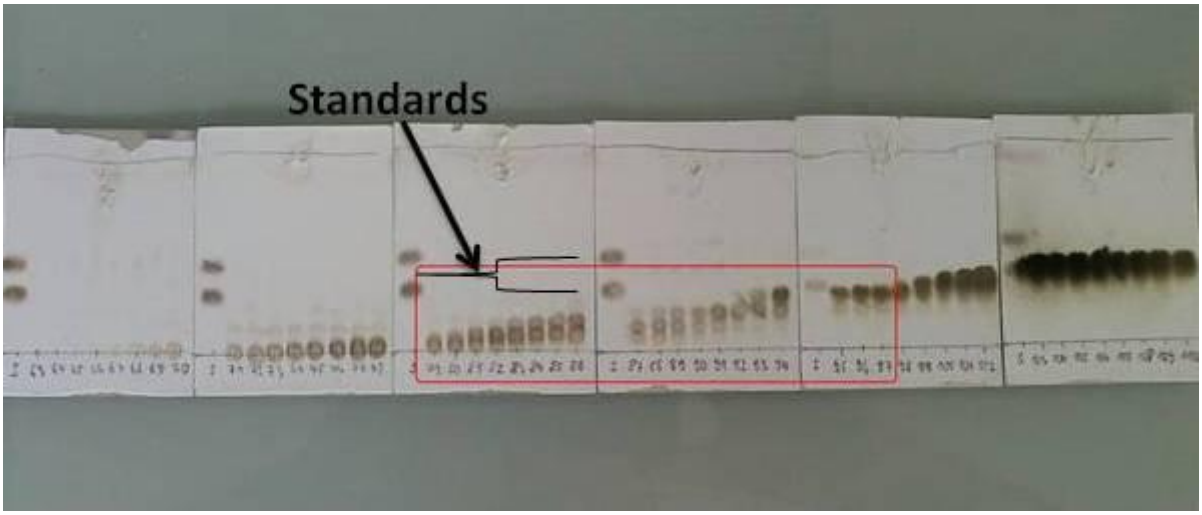


Fig. 8 TLC of human milk sample after GPC. Fractions highlighted are supposed to contain oligosaccharides, because of the different sorbent affinity than lactose and glucose (shown as standards) and previous fractions that are supposed to contain residual protein. Fractions were collected and freeze dried to obtain free oligosaccharides. In our study we detected that human milk contain higher oligosacchrides than goat and sheep milk.

5.2. Cultivation of bifidobacteria

pH values

Table (7.) and Fig. (9.) show pH values of biffidobacteria strains cultured on different carbon sources under anaerobic conditions at 37°C for 24 h. There was no growth on basal medium because pH after cultivation was from 6.5 to 6.6. Also, fucose did not support growth of bifidobacteria (pH from 5.2 to 5.9).

	Basal medium[c]	W+SP$_1$[a]	W+SP$_2$[a]	Fucose[b]	HMOs$_1$[a]	HMOs$_2$[a]
B. animalis subsp. *lactis* 1	6.6	4.8	4.7	5.9	5.5	5.8
B. animalis subsp. *lactis* 2	6.6	4.7	4.7	5.9	5.5	5.6
B. bifidum 1	6.6	5.3	5.7	5.9	4.7	4.5
B. bifidum 2	6.5	4.8	4.7	5.6	4.6	4.6
B. bifidum 3	6.5	5.1	4.9	5.5	4.6	4.6
B. longum 1	6.5	4.7	4.5	5.5	5.1	5.4
B. longum 2	6.5	5.4	4.7	5.2	4.8	4.5
Average	6.542857143[c]	4.971429[a]	4.842857[a]	5.64286[b]	4.971429[a]	5[a]

Table 7 pH values of biffidobacteria strains cultured on different carbon sources.

[a] Values are means ± standard deviation (SD) of three measurements. Values in columns with different superscript letters differ (P< 0.05). The differences among pH values were evaluated by the multiple range comparison with multiple range tests.

Values of pH were approximately similar for all carbon sources except basal medium and fucose. HMOs supported the growth of bifidobacteria and pH values were almost the same after cultivation (pH average was 5). The statistical differences among cultured media were significant (P<0.05).

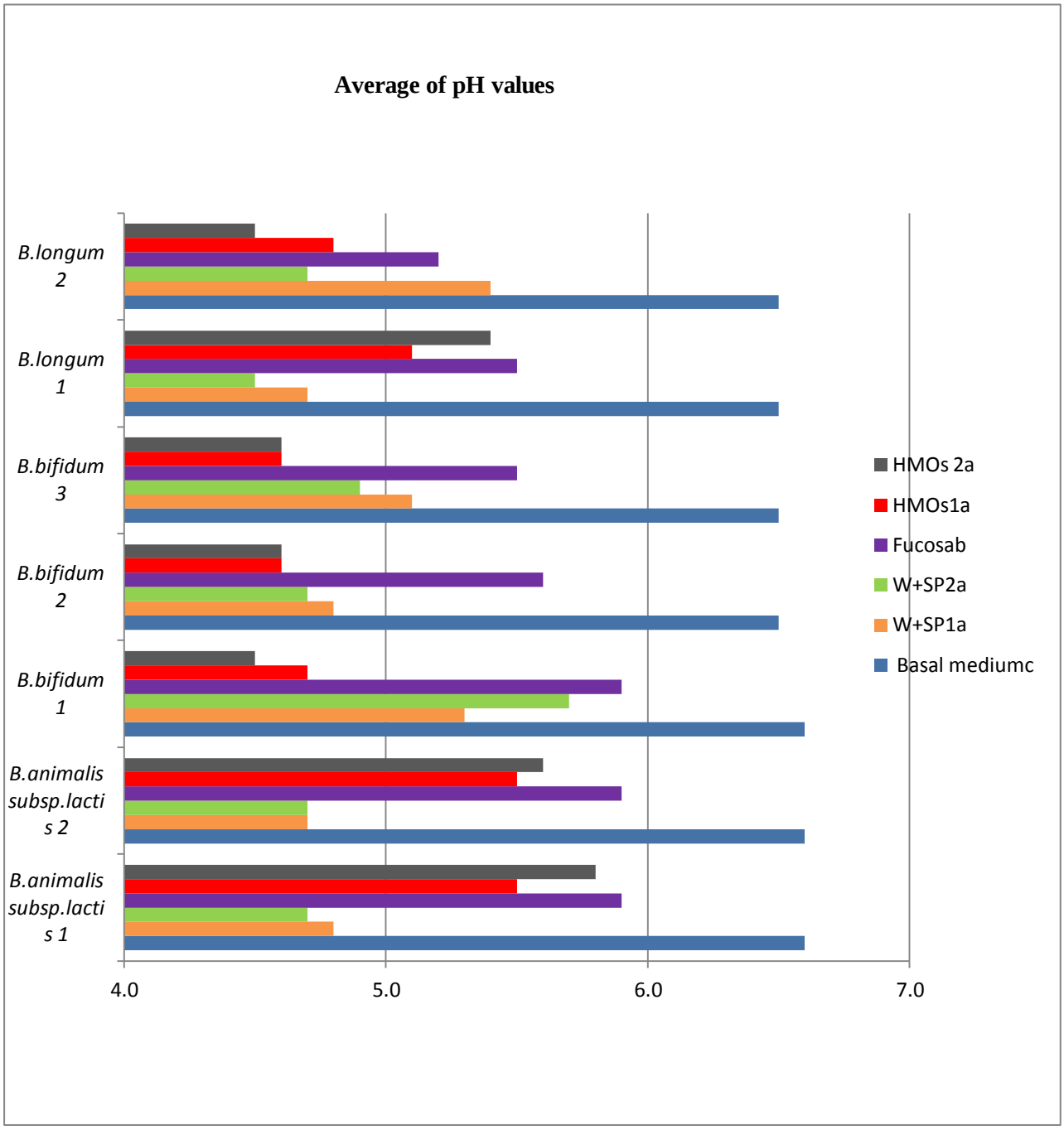


Fig. 9 **Average of pH values of biffidobacteria strains cultured on different carbon sources.**

Lactic acid values

Table (8.) and Fig. (10.) show production of lactate (mg l^{-1}) in biffidobacteria strains cultured on different carbon sources under anaerobic conditions at 37°C for 24 h.

Production of lactic acid is in line with results of pH after cultivation.

	W+SP_2[d]	Fucose[a]	HMOs $_1$[bc]	$HMOs_2$[cd]
B. animalis subsp. *lactis* 1	400	163	137	525.5
B. animalis subsp. *lactis* 2	1650	253	163.3	1035
B. bifidum 1	1340	203	1000	1655
B. bifidum 2	1605	133	985	2150
B. bifidum 3	1800	185	1805	1150
B.longum 1	750	177.5	300	345.48
B. longum 2	1170	205	370	1050
Average	1245[d]	188.5[a]	680.0429[bc]	1130.14[cd]

Table 8 Production of lactic acid by bifidobacteria strains.

[a] Values are means ± standard deviation (SD) of three measurements. Values in columns with different superscript letters differ (P< 0.05). The differences among concentration of lactic acid were evaluated by the multiple range comparison with multiple range tests.

Bifidobacteria from animal origin (*B. animalis*) produced less amount of lactic acid than bifidobacteria from human origin *(B. bifidum, B. longum)*. The highest production 2150 (mg l^{-1}) on $HMOs_2$ and the lowest production was 133 (mg l^{-1}) on fucose.

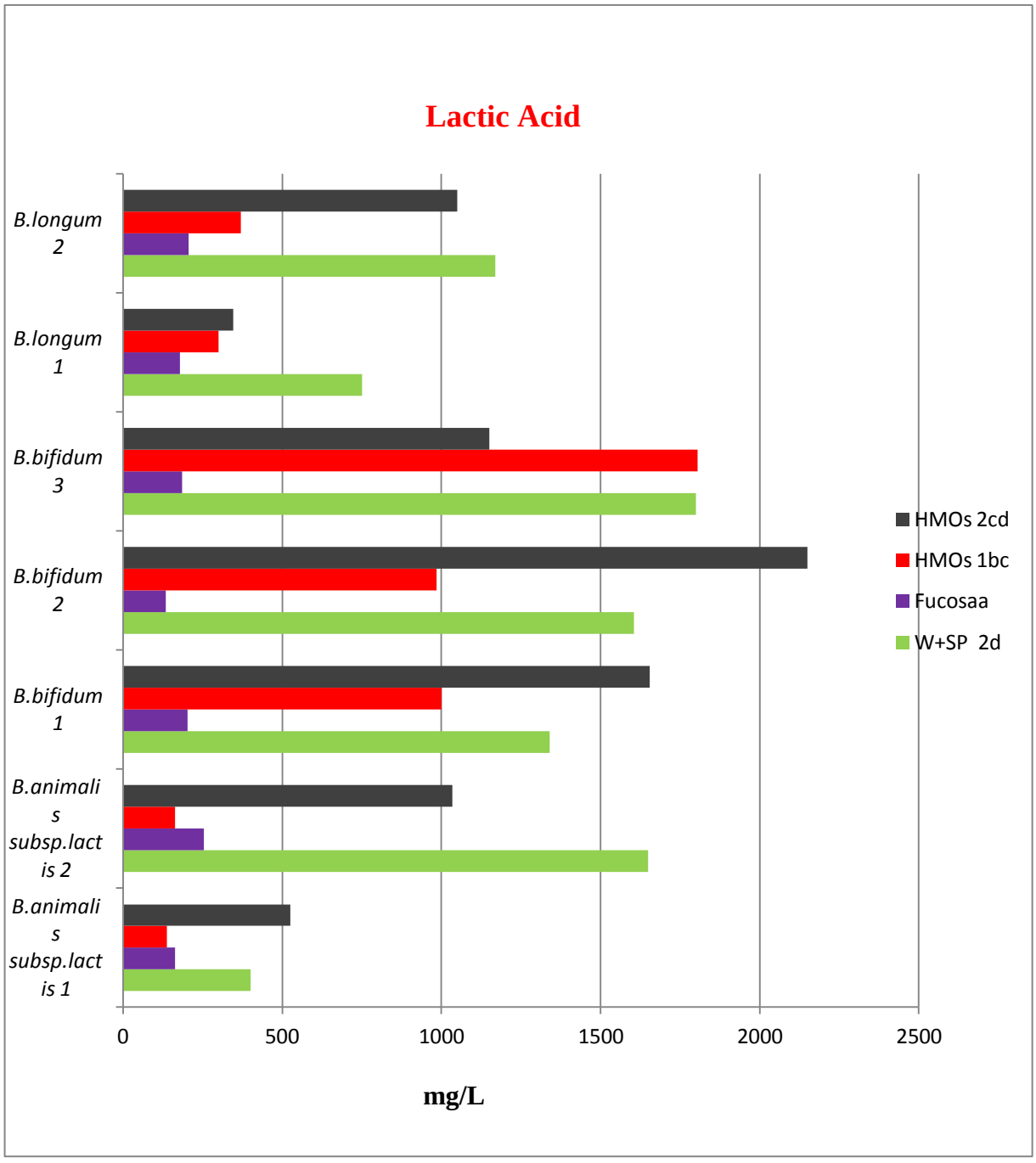


Fig. 10 Growth of bifidobacteria was in line with pH values and lactic acid production.

Growth of bifidobacteria

Table (9.) show the growth of bifidobacteria (log cfu ml^{-1}) cultivated on different sources of carbon under anaerobic conditions at 37°C for 24 h.

The best growth was seen on HMOs especially in human origin strains.

Strains	Carbone sources			
	W+SP	$HMOs_1$	fucose	$HMOs_2$
B. animalis subsp. *lactis* 1	9.77 ± 0.02^{c}	8.53 ± 0.07^{b}	7.91 ± 0.05^{a}	7.94 ± 0.12^{a}
B. animalis subsp. *lactis 2*	9.96 ± 0.05^{c}	8.60 ± 0.03^{b}	$8.00\pm0,18^{a}$	7.96 ± 0.05^{a}
B. bifidum 1	9.40 ± 0.05^{d}	8.77 ± 0.05^{b}	$7.76\pm0,10^{a}$	9.13 ± 0.07^{c}
B. bifidum 2	9.46 ± 0.03^{d}	8.10 ± 0.02^{b}	7.75 ± 0.13^{a}	8.36 ± 0.03^{c}
B. bifidum 3	9.80 ± 0.01^{d}	$8.18\pm0,08^{b}$	7.92 ± 0.16^{a}	8.91 ± 0.03^{c}
B. longum 1	9.41 ± 0.07^{d}	8.13 ± 0.02^{b}	7.70 ± 0.01^{a}	8.52 ± 0.11^{c}
B. longum 2	8.76 ± 0.28^{c}	8.01 ± 0.02^{b}	7.53 ± 0.35^{a}	8.79 ± 0.11^{c}

Table 9 The growth of bifidobacteria (log cfu ml^{-1}) cultivated on different sources of carbon.

[a] Data are means ± standard deviation (SD) of three measurements. Values in columns with different superscript letters differ (P< 0.05). The differences among bifidobacterial counts were evaluated by the multiple range comparison with multiple range tests.

The highest number of viable cells was 9.13 ± 0.07^{c} in $HMOs_2$ and the lowest number was 8.01 ± 0.02^{b} in $HMOs_1$. Fucose does not support growth of bifidobacteria.

Table (10.) shows the significance interstrains differences. The growth of bifidobacteria was different between HMOs1 and HMOs 2 in the same strain.

Strains	Carbone sources			
	W+SP	HMOs 1	fucose	HMOS 2
B. animalis subsp. *lactis* 1	9.77±0.02^{c}	8.53±0.07^{c}	7.91±0.05^{bc}	7.94±0.12^{a}
B. animalis subsp. *lactis* 2	9.96±0.05^{c}	8.60±0.03^{c}	8.00±0.18^{d}	7.96±0.05^{a}
B. bifidum 1	9.40±0.05^{b}	8.77±0.05^{d}	7.76±0.10^{abc}	9.13±0.07^{e}
B. bifidum 2	9.46±0.03^{b}	8.10±0.02^{b}	7.75±0.13^{abc}	8.36±0.03^{b}
B. bifidum 3	9.80±0.01^{c}	8.18±0.08^{c}	7.92±0.16^{bc}	8.91±0.03^{d}
B. longum 1	9.41±0.07^{b}	8.13±0.02^{b}	7.70±0.01^{ab}	8.52±0.11^{c}
B. longum 2	8.76±0.28^{a}	8.01±0.02^{a}	7.53±0.35^{a}	8.79±0.11^{d}

Table 10 The significance interstrains differences.

[a] Data are means ± standard deviation (SD) of three measurements. Values in rows with different superscript letters differ (P< 0.05). The differences among bifidobacterial counts were evaluated by the multiple range comparison with multiple range tests.

6. DISCUSSION

Bifidobacteria of human (*B. longum* and *B. bifidum*) origin grow on human milk oligosaccharides producing high quantity of lactic acid, in contrast with bifidobacteria from animal origin (*B. animalis* subsp. *lactis* 1, 2) they did not grow on human milk oligosaccharides and produced minimum amount of lactic acid. Ročková *et al.*, (2012) tested the factors affecting the growth of bifidobacteria in human milk, five strains of bifidobacteria of human origin and 2 strains of bifidobacteria of animal origin were tested for growth in 10 samples of human milk. Growth of *B. bifidum* in human milk was accompanied by a decrease in pH and production of acids. In contrast the number of viable cells of *B. animalis* was decreased from 6 log cfu ml^{-1} to 3 log cfu ml^{-1} after incubation in human milk. There were significant differences ($P < 0.05$) between bacterial counts of *B. bifidum and B. animalis* in milk samples tested. Resistance to lysozyme and the ability to utilise human milk oligosaccharides (HMOs) were identified as the most important factors affecting the growth of bifidobacteria in human milk. Four out of 5 strains of human origin were resistant to lysozyme and utilised HMOs. In contrast, *B. animalis* was susceptible to lysozyme and did not utilise HMOs. Also we found that there are differences between ability of bifidobacteria from animal origin and human origin to utilize HMOs, bifidobacteria from animal origin not allowed to utilize human milk oligosaccharides. Direct fermentation of HMOs by bifidobacteria has been poorly investigated. Ward *et al*. (2006) observsd that *B. longum* subsp. *infants* fermented HMOs, while *Lactobacillus gasseri* did not ferment HMOs. *B. longum* subsp. *infantis* preferentially consumed small mass of oligosaccharides, representing 63.9% of the total HMOs available (LoCascio *et al.*, 2007).

The same, in our experiment, *B. longum* fermented HMOs to some extent, but the most complex fermentation of HMOs was observed in *B. bifidum*, the species often found in infant gut. Bifidobacteria of human origin (*B. bifidum, B. longum*) utilized HMOs effectively, compared with bifidobacteria of animal origin (*B. animalis*).

In addition, Ročková *et al.*, (2012) reported that there are inter-species differences in the growth of bifidobacteria cultured on human milk oligosaccharides. In this study, only bifidobacteria of human origin were tested, bifidobacteria were isolated from two groups of infants. The first one (eight strains) were isolated from infants who had bifidobacteria in their faeces but, after a short period of time (4 to 24 days), bifidobacteria were no longer detected in their faeces (disappeared bifidobacteria [DB]). The second group of bifidobacteria (eight

strains) originated from infants with continual presence of bifidobacteria in their faeces (persistent bifidobacteria (PB)). There were significant differences ($p < 0.05$) between DB and PB groups in the ability of the strains to grow in HM. PB grew in HM, reaching counts higher than 7 log cfu/ml. In contrast, counts of DB decreased from 5 to 4.3 log cfu/ml after cultivation in HM. The final pH after cultivation of bifidobacteria on HMOs was 6.2 and 4.9 in DP and PB groups, respectively. In general, *Bifidobacterium bifidum* and *B. breve* species were able to utilize HMOs, while *B. adolescentis* and *B. longum* subsp. *longum* species did not. The ability to grow in HM and to utilize HMOs seems to be important properties of bifidobacteria which are able to colonize infant intestinal tract.

In our study, the differences among strains *B. longum* subsp. *longum*, *B. longum* subsp. *infantis* and *B. animalis* were tested by one-way ANOVA (Analysis of Variance) with Tukey HSD (Honestly Significant Difference) multiple comparison test ($P < 0.05$) in both tests, notably from (Table 9.) there are significant differences among the strain growth on $HMOs_1$, values in columns with different superscript letters $^{(a,b,c)}$ refers to significant difference. *B. bifidum* 1 has the highest average growth 9.13±0.07 and *B. animalis* subsp. *lactis* 1 has the lowest average growth 7.94±0.12.

There are also interstrains differences in the ability of bifidobacteria to utilize HMOs, table (10.). We found differences in bifidobacteria from animal origin the highest value is $8.53±0.07^{c}$ in $HMOs_1$ and the lowest value is $7.94±0.12^{a}$ in $HMOs_2$. Also, there are interstrain differences in bifidobacteria from human origin in *B. bifidum* 1 the highest value is $9.13±0.07^{e}$ in $HMOs_2$ and the lowest value is $8.77±0.05^{d}$ in $HMOs_1$.

Growth of bifidobacteria on human milk was accompanied by a decrease in pH (Table 7. and Fig. 9.) in some strains (pH < 5 indicates more growth), while other strains have grown well in the high pH, and pH of human milk decreases when there is high growth of bifidobacteria and pH increases when there is no growth. There are significance differences among the carbone sources, values in columns with different superscript letters $^{(a,b,c)}$ refers to significant difference in pH average between different carbon sources. For both HMOs samples there were no significant differences in pH values and it is the same between Wilkins agar and HMOs samples. There was no growth on basal medium because pH after cultivation was from 6.5 to 6.6. Also, fucose did not support growth of bifidobacteria (pH from 5.2 to 5.9).

Production of lactic acid is in line with results of pH after cultivation. Values of lactate concentration in human milk (Table 8. and Fig. 10.) are in line with data on the growth of

bifidobacteria in human milk. While *B. bifidum* is the best lactate-producing species, minimal lactate concentration was observed in *B. animalis*.

HMOs are still the best prebiotic being better than comercial available products. Bunešová *et al.*, (2012) tested the growth of infant fecal bacteria on comercial prebiotics; they tested fecal bacteria from 33 infants (aged 1 to 6 months) for growth on commercial prebiotics. The children were born vaginally or by caesarean section. Bifidobacteria, lactobacilli, gram-negative bacteria, *Escherichia coli*, and total anaerobes in fecal samples were enumerated by selective agars and fluorescence in situ hybridization. The total fecal bacteria were inoculated into cultivation media containing 2 % galacto-oligosaccharides (GOS) or fructo-oligosaccharides (FOS) as a single carbon source and bacteria were enumerated again after 24 h of anaerobic cultivation. Bifidobacteria dominated, reaching counts of 9-10 log colony-forming units (cfu)/g in 17 children born vaginally and in seven children delivered by caesarean section. In these infants, lactobacilli were more frequently detected and a lower number of *E. coli* and gram-negative bacteria were determined compared to bifidobacteria-negative infants. Clostridia dominated in children without bifidobacteria, reaching counts from 7 to 9 log CFU/g. Both prebiotics supported all groups of bacteria tested. In children with naturally high counts of bifidobacteria, bifidobacteria dominated also after cultivation on prebiotics, reaching counts from 8.23 to 8.77 log CFU/ml. In bifidobacteria-negative samples, clostridia were supported by prebiotics, reaching counts from 7.17 to 7.69 log CFU/ml. There were no significant differences between bacterial growth on GOS and FOS and counts determined by cultivation. Prebiotics should selectively stimulate the growth of desirable bacteria such as bifidobacteria and lactobacilli. However, their results showed that commercially available FOS and GOS may stimulate also other fecal bacteria.

7. CONCLUSION

Human milk oligosaccharides were isolated by GLC, fraction were screened by TLC. Fractions were collected and freeze dried to obtain free oligosaccharides. Human milk selectively stimulated the growth of specific bifidobacterial strains, bifidobacteria of human origin utilized HMOs in contrast with bifidobacteria from animal origin. Growth of bifidobacterial strains were accompanied by a decrease of pH. There were significant differences ($P < 0.05$) between bacterial counts of *B. bifidum* and *B. animalis* in milk samples tested.

8. REFERENCES

Aachary A. A., Prapulla S. G., (2008). Corncob-induced endo-1, 4-β-D-xylanase of Aspergillus oryzae MTCC 5154: production and characterization of xylobiose from glucuronoxylan. J Agric Food Chem 56 (11): 3981–88.

Abrams, S. A., Griffin, I. J., Hawthorne, K. M., Liang, L., Gunn, S. K., Darlington, G., (2005). A combination of prebiotic short- and long-chain inulin-type fructans enhances calcium absorption and bone mineralization in young adolescents. American Journal of Clinical Nutrition, 82, 471- 476.

American Academy of Pediatrics, (2005). Section on Breastfeeding. Breastfeeding and the use of human milk. Pediatrics;115:496-506.

Asahara, T., Nomoto, K., Shimizu, K., Watanuki, M., and Tanaka, R., (2001). Increased resistance of mice to *Salmonella enterica* serovar Typhimurium infection by synbiotic administration of Bifidobacteria and transgalactosylated oligosaccharides. Journal of Applied Microbiology, 91, 985-996.

Barboza, M.; Sela, D. A.; Pirim, C.; Locascio, R. G.; Freeman, S. L.; German, J. B.; Mills, D. A., and Lebrilla, C. B., (2009). Glycoprofiling bifidobacterial consumption of galacto-oligosaccharides by mass spectrometry reveals strain-specific, preferential consumption of glycans. Appl. Environ. Microbiol. 75, 7319-7325.

Beachey, E. H. (1981). Bacterial adherence: Adhesin–receptor interactions mediating the attachment of bacteria to mucosal surfaces. The Journal of Infectious Diseases, 143,325–345.

Bode, L., (2006). Recent advances on structure, metabolism, and function of human milk oligosaccharides. Journal of Nutrition, 136, 2127-2130.

Bode, L., (2009). "Human milk oligosaccharides: prebiotics and beyond". Nutrition Reviews 67(11): S183-191.

Boehm G., Stahl B., (2003). Oligosaccharides in: Mattila-Sandholm T, editor. Functional dairy products. Cambridge: Woodhead; p. 203– 243.

Boehm G., Stahl B., (2007). Oligosaccharides from milk. J Nutr.; 137: 847S–9S.

Boehm, G., Casetta, P., Jelinek, J., Lidestri, M., Negretti, F., Stahl, B., and Marini, A., (2002). Supplementation of an oligosaccharides mixture to an adapted bovine milk formula increases counts of faecal bifidobacteria in preterm infants. Arch Dis Child.

Bomba, A., Nemcova, R., Gancarcikova, S., Herich, R., Guba, P., and Mudronova, D., (2002). Improvement of the probiotic effect of microorganisms by their combination with maltodextrins, fructo-oligosaccharides and polyunsaturated fatty acids. British Journal of Nutrition, 88, 95-99.

Bordenave, S., (2000). Hydrolyse de l'alpha-lactalbumine caprine en réacteur ŕ ultrafiltration: génération et caractérisation de peptides issus de l'hydrolyse pepsique. Thčse. Université de la Rochelle, 155.

Bornet, F. R. J., Brouns, F., Tashiro, Y. and Duviller, V. (2002). Nutritional aspect of short-chain fructooligosaccharides: natural occurrence, chemistry, physiology and health implications. Digestive and Liver Disease, 34, S111–S120.

Bournet, F. R., Brouns, F., Tashiro, Y., and Duvillier, V., (2002). Nutritional aspects of short-chain fructooligosaccharides: natural occurrence, chemistry, physiology, and health implications. Digestive and Liver Disease, 34, S111-S120.

Brandtzaeg, P., (2003). Mucosal immunity: integration between mother and the breast-fed infant. Vaccine;21:3382-8.

British Nutrition Foundation. Complex carbohydrates in foods: Report of the British Nutrition's Task Force. London: Chapman and Hall; 1990.

Broek L., Hinz S. A, Beldman G., Vincken J. P., Voragen A. J. (2008). Bifidobacterium carbohydrases-their role in breakdown and synthesis of (potential) prebiotics. Mol Nutr Food Res 52 (1): 146–63.

Bugaut, M., and Bentéjac, M., (1993). Biological effects of short-chain fatty acids in nonruminant mammals. Annual Review of Nutrition, 13, 217-241.

Bunešová V., Vlková E., Rada V., knazovická V., Ročková Š., Geigerová M Božik M (2012). Growth of infant fecal bacteria on commercial prebiotics. Folia Microbiol 57:273-275.

Carlson, S. E., (1985). N-Acetylneuraminic acid concentrations in human milk oligosaccharides and glycoproteins during lactation. Am J Clin Nutr;41:720–6.

Charteris W. P., Kelly P. M., Morelli L., and Collins J. K., (1997). Selective detection, enumeration and identification of potentially probiotic Lactobacillus and

Bifidobacterium species in mixed bacterial populations. International Journal of Food Microbiology; 35: 1–27.

Ching-Ching, L., Hsueh-Fang, W., and Sheng-Dun, L., (2008). Effect of Isomalto-oligosaccharide syrup on quality characteristics of sponge cake. Cereal Chem. 85(4): 515–521.

Coppa, G. V., Gabrielli, O., Pierani, P., Catassi, C., Carlucci, A. and Giorgi, P. L. (1993) Changes in carbohydrate composition in human milk over 4 months of lactation. Pediatrics 91, 637-641.

Crittenden, R., Karppinen, S., Ojanen, S., Tenkanen, M., Fagerström, R., Mättö, J., Saarela, M., Mattila-Sandholm, T. and Poutanen, K., (2002). In vitro fermentation of cereal dietary fibre carbohydrates by probiotic and intestinal bacteria. Journal of the Science of Food and Agriculture 82: 781-789.

Delzenne, N. M., Daubioul, C., Neyrinck, A., Lasa, M., and Taper, H. S., (2002). Inulin and oligofructose modulate lipid metabolis in animals, review of biological events and future prospects. British Journal of Nutrition, 87 (Suppl. 1), 255-259.

Demigné, C., Jacobs, H., Moundras, C., Davicco, M. J., Horcajada, and M. N., Bernalier, (2008). Comparison of native or reformulated chicory fructans, or nonpurified chicory, on rat cecal fermentation and mineral metabolism. European Journal of Nutrition, 47, 366- 374.

Demuth, K., Jordening, H. J., and Buchholz, K., (2002). Oligosaccharide synthesis by dextransucrase: new unconventional acceptors. Carbohyd. Res. 337: 1811–1820.

Dixon B., (2002). Secrets of the Bulgarian bacillus. Lancet Infect Dis.; 2:260.

Dewey, K. G., Peerson, J. M., Brown, K. H., Krebs, N. F., Michaelsen, K. F., Persson, L. A., Salmenpera, L., Whitehead, R. G., and Yeung, D. L., (1995). Growth of breast-fed infants deviates from current reference data: a pooled analysis of US, Canadian, and European data sets. World Health Organization Working Group on Infant Growth. Pediatrics 96, 495–503.

Dinoto, A.; Marques, T. M.; Sakamoto, K.; Fukiya, S.; Watanabe, J.; Ito, S., and Yokota, A., (2006). Population dynamics of Bifidobacterium species in human faeces during raffinose administration monitored by fluorescence in situ hybridization-flow cytometry. Appl. Environ. Microbiol. 72, 7739-7747.

Djouzi, Z. and Andrieux, C., (1997). Compared effects of three oligosaccharides on metabolism of intestinal microflora in rats inoculated with a human fecal flora. Brit. J Nutr. 78: 313–324.

Djouzi, Z., Andrieux, C., Pelenc, V., Somarriba, S., Popot, F., Paul, F., Monsan, P., and Szylit, O., (1995). Degradation and fermentation of α-glucooligosaccharides by bacterial strains from human colon – in vitro and in vivo studies in gnotobiotic rats. J Appl Bact. 79 (2): 117–127

Domínguez, H., Alonso, J. L., Garrote, G., Parajó, J. C. and Vázquez, M. J., (2003). Xylo oligosaccharides: properties and production technologies. Electronical Journal of Environmental, Agricultural and Food Chemistry 2: 230-232.

Erney, R. M., Malone, W.T., Skelding, M. B., Marcon, A. A., Klemen-Leyer, K. M., O'Ryan, M. L., (2000). Variability of human milk neutral oligosaccharides in a diverse population. J Pediatr Gastroenterol Nutr; 30: 181-92.

Ezeh, V. N., and Lewis, M. J., (2011). Milk reversibility following reduction and restoration of pH. International Journal of Dairy Technology; 64(2):179-87.

FAO/WHO (2002). Guidelines for the evaluation of probiotics in food. London, Ontario: Food and Agriculture Organization of the United Nations and World Health OrganizationWorking Group Report, 1e11.

FAO, (2003). ProductionYearbook 2002. Food Agric. Organisation, UN, Rome, Italy, p. 271.

Fanaro, S.; Boehm, G.; Garssen, J.; Knol, J.; Mosca, F., and Stahl, B., (2005). Galactooligosaccharides and long-chain fructo-oligosaccharides as prebiotics in infant formulas: a review. Acta Paediatrica Supplement, 94, 22-26.

Fergusson, D. M., Beautrais, A. L., and Silva, Pas. (1982). Breast-feeding and cognitive development in the first seven years of life. Soc. Sci. Med. 16, 1705–1708.

Fiordaliso, M., Kok, N., Desager, J. P., Goethals, F., Deboyser, D., Roberfroid, M., (1995). Dietary oligofructose lowers triglycerides, phospholipids and cholesterol in serum and very low density lipoproteins of rats. Lipids, 30, 163-167.

Flamm G, W. Glinsmann, D. Kritchevsky, L. Prosky and M. B. Roberfroid, (2001). "Inulin and Oligofructose as Dietary Fiber: A Review of the Evidence," Critical Reviews in Food Science and Nutrition, Vol. 41, No. 5, , pp. 353-362. doi:10.1080/20014091091841.

Fooks L. J., Gibson G. R., (2002). In vitro investigations of the effect of probiotics and prebiotics on selected human intestinal pathogens. FEMS Microbiol Ecol 39 (1): 67–75.

Forchielli, M. L., & Walker, W. A., (2005). The role of gut-associated lymphoid tissues and mucosal defence. British Journal of Nutrition, 93 (Suppl. 1), 41-48.

Fuller, R., (1992a). Probiotics: The Scientific Basis. Chapman & Hall, London.

Fuller, R., (1992b). The efects of probiotics on the gut microecology of farm animals. In the actic acid bacteria, Vol. 1, ed. B. J. B. Wood. Chapman & Hall, London, pp. 171Ð192.

Fuller, R., (1997). Probiotics 2: Applications and Practical Aspects. Chapman & Hall, London.

Garrido, D.; Barile, D., and Mills, D. A., (2012a). A molecular basis for bifidobacterial enrichment in the infant gastrointestinal tract. Adv. Nutr. 3, 415S-421S.

Garrido, D.; Nwosu, C.; Ruiz-Moyano, S.; Aldredge, D.; German, J. B.; Lebrilla, C. B., and Mills, D. A., (2012b). Endo-beta-N-acetylglucosaminidases from infant gutassociated bifidobacteria release complex n-glycans from human milk glycoproteins. Mol. Cell. Proteomics, 11, 775-785.

Gaucheron, F., (2005). The minerals of milk. Reprod Nutr Dev;45(4):473-83.

Gibson, G. R., and Roberfroid, M. B., (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. Journal of Nutrition 125, 1401:1412.

Gibson G. R., and Wang, X., (1994). Bifidogenic properties of different types of fructooligosaccharides‖ . Food Microbiology; 11: 491-498.

Gibson G. R., Probert H. M., Van Loo J., Rastall R. A. , Roberfroid M. B., (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutrition Research Reviews, 17:259-275.

Gilad, O.; Jacobsen, S.; Stuer-Lauridsen, B.; Pedersen, M. B.; Garrigues, C., and Svensson, B., (2010). Combined transcriptome and proteome analysis of Bifidobacterium animalis subsp. lactis BB-12 grown on xylo-oligosaccharides and a model of their utilization. Appl. Environ. Microbiol. 76, 7285-7291.

Gopal, P. K., and Gill, H. S., (2000). Oligosaccharides and glycoconjugates in bovine milk and colostrum. British Journal of Nutrition, 84(Suppl 1), S69–S74.

Goulas, T.; Goulas, A.; Tzortzis, G., and Gibson, G. R., (2009a). Comparative analysis of four beta-galactosidases from Bifidobacterium bifidum NCIMB41171: purification and biochemical characterisation. Appl. Microbiol. Biotechnol. 82, 1079-1088.

Graham H., Apajalahti J., Peuranen S., (2004). Xylooligosaccharides alter metabolism of gut microbes and blood xylose levels in chicks. In: van der Kamp JW, Asp NG, Miller Jones J, Schaafsma G, editors. Dietary fiber:bioactive carbohydrates for food and feed. The Netherlands: Wageningen Academic Publishers. p 329–32.

Greco, L., Auricchio S., Mayer M., Grimald M., (1998). Case control study on nutritional risks in celiac disease. J Pediatr Gastroenterol Nutr;7:395-9.

Guéguen, L., (1997). La valeur nutritionnelle minérale du lait de chčvre In: Intérets nutritionnel et diététique du lait de chčvre, Niort, Ed INRA, Paris Colloques 7 nov 1996, pp. 67–80.

György P, Norris RF, Rose CS. Bifidus factor I. A variant of Lactobacillus bifidus requiring a special growth factor. Arch Biochem Biophys 1954;48:193–01.

Haenlein, G. F. W., and Wendorff, W., (2006). Sheep milk. In: Park, Y.W., Haenlein, G.F.W. (Eds.), Handbook of Milk of Non-bovine Mammals. Blackwell Publishing Professional, Oxford, England, pp. 137–194.

Hagel, L. (2001). "Gel-filtration chromatography." Curr Protoc Mol Biol. Chapter 10: Unit 10 19.

Hamilton-Miller, J. M. T., Gibson, G. R., and Bruck, W., (2003). Some insight into the derivation and early uses of the word ‘probiotic’. British Journal of Nutrition, 90, 845.

Harmsen, H. J. M., Wildeboer-Veloo, A.C. M., Raangs, G. C., (2000). Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J Pediatr Gastroenterol Nutr; 30:61–67.

Hayashi, S., T. Honitani, and K. Imada., (1994). The enzymatic reaction for the production of panose and isomaltose by glucosyltransferase from Aureobasidium. Lett. Appl. Microbiol. 19:247–252.

Heinig, M. J., (2001). Host defense benefits of breastfeeding for the infant. Effect of breastfeeding duration and exclusivity. Pediatr Clin North Am;48:105-23.

Hickey, R. M., (2009). Harnessing milk oligosaccharides for nutraceutical applications. In M. Corredig (Ed.), Dairy derived ingredients. Food and nutraceutical uses (pp. 308-343). London, UK: Woodhead Publishing.

Hinz, S. W.; Pastink, M. I.; van den Broek, L. A.; Vincken, J. P., and Voragen, A. G., (2005). Bifidobacterium longum endogalactanase liberates galactotriose from type I galactans. Appl. Environ. Microbiol. 71, 5501-5510.

Holt, C., Dalgleish, D. G., and Jenness, R., (1981). Calculation of the ion equilibria in milk diffusate and comparison with experiment. Anal Biochem. 1;113(1):154-63.

Holt, C., and Jenness, R., (1984). Interrelationships of constituents and partition of salts in milk samples from eight species. Comp. Biochem. Physiol. 77A (2), 275–282.

Hsu C, Liao J. W., Chung Y. C., Hsieh C. P., and. Chan Y. C., (2004). "Xylooligosaccharides and Frutooligosaccharides Affect the Intestinal Microbiota and Precancerous Colonic Lesion Development in Rats," *The Journal of Nutrition*, Vol. 134, No. 6, , pp. 1523-1528.

Imaizumi, K., Nakatsu, Y., Sato, M., Sedarnawati, Y. and Sugano, M. (1991). Effects of xylooligosaccharides on blood glucose, serum and liver lipids and caecum short-chain fatty acids in diabetic rats. Agriculture Biology and Biochemistry, 55, 199–205.

Jaubert, A., (1997). Les vitamines et les nucléotides du lait de chčvre. In: Intérets nutritionnel et diététique du lait de chčvre, Ed INRA, Paris, Colloques 7 nov 1996, pp. 81–92.

Joint IUB-IUPAC (1997). Commission on Biochemical Nomenclature (JCBN). Nomenclature of carbohydrates, recommendations 1996. Carbohydr Res.;297:1–92.

Kaneko, T., A. Yokoyama, and M. Suzuki, (1995). Digestibility characteristics of isomaltooligosaccharides in comparison with several saccharides using the rat jejunum loop method. Biosci. Biotechnol. Biochem. 59:1190–1194.

Karlsson, K. A. (1995). Microbial recognition of target-cell glycoconjugates. Current Opinion in Structural Biology, 5, 622–635.

Katapodis P., Christakopoulos P., (2005). Xylanases as a tool for the production of novel phytopharmaceuticals. NutraCos 4:17–21.

Katapodis P., Kavarnou A., Kintzios S., Pistola E., Kekos D., Macris B. J., Christakopoulos P., (2002). Production of acidic xylooligosaccharides by a family 10 endoxylanase from *Thermoascus aurantiacus* and use as plant growth regulators. Biotechnol Lett 24:1413–6.

Kawasaki, S.; Mimura, T.; Satoh, T.; Takeda, K., and Niimura, Y., (2006). Response of the microaerophilic Bifidobacterium species, B. boum and B. thermophilum, to oxygen. Appl Environ Microbiol., 72:6854–6858.

Kim, Y. S., Tsa, O. D., Morita, A., and Bella, A., (1982). Effect of sodium butyrate and three human colorectal adenocarcinoma cell lines in culture. Falk Symposium, 31, 317-323.

Kohmoto T., Fujui F., Takaku H., Mitsuoka T., (1991). Dose-response test of isomaltooligosaccharides for increasing fecal Bifidobacteria. Agric Biol Chem 55:2157–2159.

Kohmoto T., Tsuji K., Kaneko T., Shiota M., Fukui F., Takaku H., Nakagawa Y., Ichikawa T., Kobayashi S., (1992). Metabolism of 13 Cisomal to oligosaccharides in healthy men. Biotech Biochem 56:937–940.

Kokubo I., Ikemizu S., (2004). Histamine-release inhibitors containing xylooligosaccharides. Japan Patent JP 2004059481:2004.

Kunz, C., and S. Rudloff, (1993). "Biological Functions of Oligosaccharides in Human-Milk". Acta Paediatrica 82(11): 903-912.

Kunz, C., and Rudloff, S. (2006). Health promoting aspects of milk oligosaccharides. International Dairy Journal, 16, 1341-1346.

Kuntz, S., Rudloff, S. and Kunz, C., (2008) Oligosaccharides from human milk influence growth-related characteristics of intestinally transformed and non-transformed intestinal cells. Br. J. Nutr. 99, 462-471.

Kunz, C., Rudloff, S., Schad, W., and Braun, D., (1999). Lactose-derived oligosaccharides in the milk of elephants: Comparison with human milk. British Journal of Nutrition, 82(5), 391–399.

Kunz, C.; Rudloff, S.; Baier, W.; Klein, N., and Strobel, S., (2000). Oligosaccharides in human milk: structural, functional, and metabolic aspects. Annual Reviews in Nutrition, 20, 699-722.

Lagaert, S.; Pollet, A.; Delcour, J. A.; Lavigne, R.; Courtin, C. M., and Volckaert, G., (2010). Substrate specificity of three recombinant alpha-L-arabinofuranosidases from Bifidobacterium adolescentis and their divergent action on arabinoxylan and arabinoxylan oligosaccharides. Biochem. Biophys. Res. Commun. 402, 644-650.

Langlands, S. J., Hopkins, M. J., Coleman, N., & Cummings, J. H., (2004). Prebiotic carbohydrates modify the mucosa associated microflora of the human large bowel. Gut, 53, 1610-1616.

Lawrence, R. M., & Pane, C. A. (2007). Human breast milk: current concepts of immunology and infectious diseases. Current Problems in Pediatric and Adolescent Health Care, 37, 7-36.

Lee, J. H., and O'Sullivan, D. J., (2010). Genomic insights into bifidobacteria. Microbiol. Mol. Biol. Rev. 74, 378-416.

Lilly D. M., Stillwcll R. H., (1965). Probiotics growth promoting factors produced by micro-organisms. Science;147:747-8.

LoCascio, R. G., Ninonuevo, M. R., Freeman, S. L., Sela, D. A., Grimm, R., Lebrilla. C. B., (2007). Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. J Agric Food Chem; 55: 8914–9.

LoCascio, R. G., Niñonuevo, M. R., Kronewitter, S. R., Freeman, S. L., German, J. B., Lebrilla, C. B., Mills, D. A., (2009). A versatile and scalable strategy for glycoprofiling bifidobacterial consumption of human milk oligosaccharides. Microbial Biotechnology, 2, 333–342.

Lomax A. R., Calder P. C., (2009). Prebiotics, immune function, infection and inflammation: a review of the evidence. British Journal of Nutrition, 101:633-658.

Lönnerdal, B., (1997). Effects of milk and milk components on calcium, magnesium, and trace element absorption during infancy. Physiol Rev; 77: 643–69.

Lucas, A., Morley, R. and Cole, T. J., (1998). Randomised trial of early diet in preterm babies and later intelligence quotient. BMJ 317, 1481–1487.

Lucas, A., Morley, R., Cole, T. J., Lister, G., and Leeson-Payne, C., (1992). Breast milk and subsequent intelligence quotient in children born preterm. Lancet 339, 261–264.

Lundequist, B.; Nord, C. E., and Winberg, J., (1985). The composition of the faecal microflora in breastfed and bottle fed infants from birth to eight weeks. Acta Paediatr Scand 74:45-51.

Macfarlane G. T., Steed H., Macfarlane S., (2008). Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. J Appl Microbiol 104:305–44.

Marcobal, A.; Barboza, M.; Froehlich, J. W.; Block, D. E.; German, J. B.; Lebrilla, C. B., and Mills, D. A., (2010). Consumption of human milk oligosaccharides by gut-related microbes. J. Agric. Food Chem. 58, 5334-5340.

Marteau P, Cuillerier E, Meance S., (2002). Bifidobacterium animalis strain DN-173 010 shortens the colonic transit time in healthy women: a double-blind, randomized, controlled study. Alimentary Pharmacology and Therapeutics; 16: 587–593.

Menkes, J. H., (1977). Early feeding history of children with learning disorders. Dev. Med. Child Neurol. 19, 169–171.

Mikkelsen, L. L., and Jensen, B. B., (2004). Effect of fructo-oligosaccharides and transgalacto oligosaccharides on microbial populations and microbial activityin the gastrointestinal tract of piglets post-weaning. Animal Feed Science and Technology, 117, 107–119.

Mitsuoka, T., Hidaka, H. and Eida, T. (1987) Effect of fructooligosaccharides on intestinal microflora. Die Nahrung, 31, 427–436.

Modler, H. W. (1994) Bifidogenic factors – sources, metabolism and applications. International Dairy Journal, pp. 383–407.

Morgan, B. L. G., and Winick, M., (1980). Effects of administration of N-acetylneuraminic acid (NANA) on brain NANA content and behavior. J Nutr;110:416–24.

Morgan, A. J., Mul, A. J., Beldman, G. and Voragen, A. G. J. (1992). Dietary oligosaccharides – new insights. AGRO Food Industry High Technology, Nov/Dec, 35–38.

Mortensen, E. L., Michaelsen, K. F., Sanders, S. A., and Reinisch, J. M., (2002). The association between duration of breastfeeding and adult intelligence. JAMA 287 (18), 2365–2371.

Mountzouris, K. C., Gilmour, S. G., and Rastall, R. A., (2002). Continuous production of oligodextrans via controlled hydrolysis of dextran in an enzyme membrane reactor. J Food Sci., 67(5): 1767–1771.

Mussatto S. I., Mancilha I. M., (2007). Non-digestible oligosaccharides: a review. Carbohyd Polym 68:587–597.

Nakano H., (1998). "Recent Japanese Development in the Enzymatic Production and Application of Oligosaccharides," Presented at the Seminar on Enzyme and Bacterial Technology , Campinas. Japan International Cooperation Agency (s.d).

Newburg, D. S.; Ruiz-Palacios, G. M., and Morrow, A. L., (2005). Human milk glycans protect infants against enteric pathogens. Annual Reviews in Nutrition, 25, 37-58.

Niñonuevo, M. R., Ward, R. E., and LoCascio, R.G., (2007). Methods for the quantitation of human milkoligosaccharides in bacterial fermentation by mass spectrometry. Anal Biochem; 361:15–23.

O'Connell Motherway, M.; Fitzgerald, G. F., and van Sinderen, D., (2011). Metabolism of a plant derived galactose-containing polysaccharide by *Bifidobacterium breve* UCC2003. Microb. Biotechnol. 4, 403-416.

Ofek, I., & Sharon, N. (1990). Adhesins as lectins: Specificity and role in infection. Current Topics in Microbiology and Immunology, 151, 91–114.

Ogawa, K.; Ben, R. A., and Pons, S., (1992). Volatile fatty acids, lactic acid, and pH in the stools of breast-fed and bottle-fed infants. J Pediatr Gastroenterol Nutr 15:248-252.

Omori, T.; Ueno, K.; Muramatsu, K.; Kikuchi, M.; Onodera, S., and Shiomi, N., (2010). Characterization of recombinant beta-fructofuranosidase from Bifidobacterium adolescentis G1. Chem. Cent. J. 4, 9.

Paccard, P., and Lagriffoul, G., (2006a). Synthčse bibliographique sur la composition du lait de brebis en composés d'intéręt nutritionnel. Personal communication, 28 pp.

Paccard, P., and Lagriffoul, G., (2006b). Synthčse bibliographique sur la composition des fromages de brebis en composés d'intéręt nutritionnel. Personal communication, 24 pp.

Palma, A. S., Feizi, T., Zhang, Y., Stoll, M. S., Lawson, A. M., Díaz-Rodríguez, E., et al. (2006). Ligands for the beta-glucan receptor, Dectin-1, assigned using "designer" microarrays of oligosaccharide probes (neoglycolipids) generated from glucan polysaccharides. Journal of Biological Chemistry, 281, 5771-5779.

ParajoJ. C, Garrote G., Cruz J. M., and Dominguez H., (2004). "Production of Xylooligosaccharides by Autohydrolysis of Lignocellulosic Materials," Trends in Food Science and Technology, Vol. 15, No. 3-4, pp. 115-120. doi:10.1016/j.tifs.2003.09.009.

Park, Y. W , Juarez, M., Ramos, M., and Haenlein, G. F. W., (2007). Physico-chemical characteristics of goat and sheep milk. Science Direct. Small Ruminant Research 68:88−113.

Parker, R. B., (1974). Probiotics, the other half of the story. Animal Nutrition and Health, 29, 4–8.

Paul, F., Lopez-Munguia, A., Remaud, M., Pelenc, V., and Monsan, P., (1992). Method for the production of α-(1, 2) oligodextrans using Leuconostocmesenteroides B-1299. US Patent 5141858.

Perrin, S.; Warchol, M.; Grill, J. P., and Schneider, F., (2001). Fermentations of fructooligosaccharides and their components by Bifidobacterium infantis ATCC 15697 on batch culture in semi-synthetic medium. J. Appl. Microbiol. 90, 859-865.

Perrin S., Fougnies C., Grill J. P., Jacobs H., Schneider F., (2002). Fermentation of chicory fructo-oligosaccharides in mixtures of different degrees of polymerization by three strains of bifidobacteria. Can J Microbiol.; 48: 759–63.

Playne, M. J., and Crittenden, R. G., (2004). Part II Biotechnology strategies for producing specific food ingridents. In: Bioprocesses and Biotechnology for Functional Foods and Nutraceuticals, pp. 120–121, Neeser, J.R. and German, J.B., Eds., CRC Press, Boca Raton, FL.

Pokusaeva, K.; O'Connell-Motherway, M.; Zomer, A.; Macsharry, J.; Fitzgerald, G. F., and van Sinderen, D., (2011). Cellodextrin utilization by *Bifidobacterium breve* UCC2003. Appl. Environ. Microbiol. 77, 1681-1690.

Prenosil, J. E., Stuker, E. and Bourne, J. R., (1987). Formation of oligosaccharides during enzymic lactose hydrolysis. I. State of the art. Biotechnol Bioeng 30, 1019–1025.

Rada, V., Splíchal, I., Rocková, S., Grmanová, M., & Vlková, E. (2010). Susceptibility of bifidobacteria to lysozyme as a possible selection criterion for probiotic bifidobacterial strains. Biotechnology Letters, 32, 451e455.

Raynal-Ljutovac, K., Lagriffoul, G., Paccard, P., Guillet, I., and Chilliard, Y., (2008). Composition of goat and sheep milk products: An update Small Ruminant Research 79: 57–72.

Reimer, R. A., & Russell, J. C., (2008). Glucose tolerance, lipids, and GLP- secretion in JCR: LA-cr Ratf Fed h Higp Proteif Fibed Diet. Obesity, 16, 40-46.

Remaud-Simeon, M., Lopez-Munguia, A., Pelec, V., Paul, F. and Monsan, P., (1994). Production and use of glucosyltransferases from Leuconostocmesenteroides NRRL B-1299 for the synthesis of oligosaccharides containing α- (1,2) linkages. Appl Biochem Biotechnol. 44: 101–117.

Reuter, G., (1963). Vergleichende Untersuchunge u¨ber die Bifidus-Flora im Sa¨uglings- und Erwachsenenstuhl. Zentralbl Bakteriol Orig, 191, 486–507 (in German).

Roberfroid M. B., (1993). "Dietary Fiber, Inulin, and Oligofructose: A Review Comparing Their Physiological Effects," Critical Reviews in Food Science and Nutrition, Vol. 33, No. 2, , pp. 103-148. doi:10.1080/10408399309527616.

Roberfroid M. B., (2007). Inulin-type fructans: functional food 7. ingredients. J Nutr;137:2493S-2502S.

Roberfroid, M. B., and Delzenne, N., (1998). Dietary fructans. Annual Review of Nutrition; 18: 117–43.

Roberfroid, M. B., Van Loo, J., and Gibson, G. R., (1998). The bifidogenic nature of chicory inulin and its hydrolysis products. Journal of Nutrition; 128: 11–9.

Ročková Š., Nevoral J., Rada V., Maršík P., Sklenář J., Hlinková A., Vlková E., Marounek M. (2011). Factors affecting the growth of bifidobacteria in human milk. International Dairy Journal 21 (2011) 504-508.

Ročková Š., Rada V., Nevoral J., Maršík P.,Vlková E., Bunesove V (2012). Inter-species differences in the growth of bifidobacteria cultured on human milk oligosaccharides Folia Microbiol 57:321-324.

Ročková, Š., Rada.,V, Maršík, P.; Vlková, E.; Bunesova, V.; Sklenar, J., and Splichal, I., (2006). Growth of bifidobacteria and clostridia on human and cow milk saccharides. Anaerobe, 17: 223-225.

Rodgers, B., (1978). Feeding in infancy and later ability and attainment: a longitudinal study. Dev. Med. Child Neurol. 20, 421–426.

Rossi, M.; Corradini, C.; Amaretti, A.; Nicolini, M.; Pompei, A.; Zanoni, S., and Matteuzzi, D., (2005). Fermentation of fructooligosaccharides and inulin by bifidobacteria: a comparative study of pure and fecal cultures. Appl. Environ. Microbiol. 71, 6150-6158.

Ruas-Madiedo, P.; Gueimonde, M.; Fernandez-Garcia, M.; de los Reyes-Gavilan, C. G., and Margolles, A., (2008). Mucin degradation by Bifidobacterium strains isolated from the human intestinal microbiota. Appl. Environ. Microbiol. 74, 1936-1940.

Salminen, S. and Salminen, E. (1997). Lactulose, lactic acid bacteria, intestinal microecology and mucosal protection. Scandinavian Journal of Gastroenterology, 32 (Suppl.), 45–48.

Salminen S., Bouley C., Boutron-Ruault M. C., (1998). Functional food science and gastrointestinal physiology and function. British Journal of Nutrition; 80(supplement 1): S147–S171.

Scheppach, W., and Weiler, F., (2004). The butyrate story: old wine in new bottles? Butyrate appears to be essential for a wide range of intestinal mucosal health benefits; however, the mechanisms behind this remain to be determined. Current Opinion in Clinical Nutrition Metabolic Care, 7, 563-567.

Scheppach, W., Bartram, P., Richter, A., Richter, F., Liepold, H., Dusel, G., (1992). Effect of short-chain fatty acids on the human colonic mucosa in vitro. Journal of Parenteral and Enteral Nutrition, 16(1), 43-48.

Schiffrin, E. J., and Blum, S., (2002). Interactions between the microbiota and the intestinal mucosa. Eur J Clin Nutr 56:S60-S64.

Scholz-Ahrens, K. E., and Schrezenmeir, J., (2007). Inulin and oligofructose and mineral metabolism: the evidence from animal trials. Journal of Nutrition, 137(11 Suppl.), 2513S - 2523S.

Sela, D. A., Mills, D. A., (2010). Nursing our microbiota: molecular linkages between bifidobacteria and milk oligosaccharides. Trends in Microbiology, 18, 298–307.

Sela, D. A.; Chapman, J.; Adeuya, A.; Kim, J. H.; Chen, F.; Whitehead, T. R.; Lapidus, A.; Rokhsar, D. S.; Lebrilla, C. B.; German, J. B.; Price, N. P.; Richardson, P. M., and Mills, D. A., (2008). The genome sequence of *Bifidobacterium longum* subsp *infantis* reveals adaptations for milk utilization within the infant microbiome. Proceedings of the National Academy of Sciences of the United States of America, 105, 18964–18969.

Sinkiewicz, G., Nordstrom, E. A., (2005). Occurence of Lactobacillus reuteri, lactobacilli and bifidobacteria in human breast milk. Pediatr. Res. 58, 415.

Smith, M. M., Durkin, M., Hinton, V. J., Belinger, D., & Kuhn, L. (2003). Influence of breastfeeding on cognitive outcomes at age 6-8 years: follow-up of very low birth weight infants. American Journal of Epidemiology, 158, 1075-1082.

Takaku, H., (1988). Anomalously linked oligosaccharides mixture in: Handbook of Amylases and Related Enzymes: Their Sources, Isolation Methods, Properties and Applications. pp. 215–217. The Amylase Research Society of Japan, Osaka, Japan, Ed., Pergamon Press, New York.

Tako, E., Glahn, R. P., Welch, R. M., Lei, X., Yasuda, K., and Miller, D. D., (2008). Dietary inulin affects the expression of intestinal enterocyte iron transporters, receptors and storage protein and alters the microbiota in the pig intestine. British Journal of Nutrition, 99, 472- 480.

Tamine, A., Marshall V., and R. Robinson, (1995). "Microbiological and Technological Aspects of Milks Fermented by Bifidobacteria," Journal of Dairy Research, Vol. 62, No. 1, , pp. 151-187.

Teitelbaum J. E., Walker W. A., (2002). Nutritional impact of preand probiotics as protective gastrointestinal organisms. Annu Rev Nutr 22:107–138.

Teuri, U. and Korpel, R. (1998). Galacto-oligosaccharides relieve constipation in elderly people. Annals of Nutrition and Metabolism, 42, 319–327.

Urashima, T., Saito, T., Nakamura, T., and Messer, M., (2001). Oligosaccharides of milk and colostrum in non-human mammals. Glycoconjugate Journal, 18(5), 357–371.

Valéria Maria, Caselato de Sousa, Elisvânia Freitas dos Santos, and Valdemiro Carlos Sgarbieri (2011). The Importance of Prebiotics in Functional Foods and Clinical Practice Food and Nutrition Sciences, 2011, 2, 133-144.

Van Laere, K. M.; Beldman, G., and Voragen, A. G., (1997). A new arabinofuranohydrolase from Bifidobacterium adolescentis able to remove arabinosyl residues from double-substituted xylose units in arabinoxylan. Appl. Microbiol. Biotechnol. 47, 231-235.

Vazquez M. J., Alonso J. L., Dominguez H., (2000). Xylooligosaccharides: manufacture and applications. Trends Food Sci Technol 11, 387–393.

Ventura, M.; Canchaya, C.; Tauch, A.; Chandra, G.; Gerald, F.; Fitzgerald; Keith, F.; Chater, and Douwe van, S. (2007). Genomics of *Actinobacteria*: tracing the evolutionary history of an ancient phylum. Microbiol Mol Biol Rev.;71:495–548.

Vernazza, C. L.; Gibson, G. R., and Rastall, R. A., (2005). In vitro fermentation of chitosan derivatives by mixed cultures of human faecal bacteria. Carbohydrate Polymers, 60, 539−545.

Vetere, A., A. Gamini, C. Campa, and S. Paoletti. (2000). Regiospecific transglycolytic synthesis and structural characterization of 6-o-alpha-glucopyranosyl-glucopyranose (isomaltose). Biochem. Biophys. Res. Commun. 274:99–104.

Walker, W. A., (2004). The dynamic effects of breastfeeding on intestinal development and host defense. Adv Exp Med Biol;554:155-70.

Ward E., Ninonuevo, m Mills A., Lebrilla B., and Bruce J.. (2006) In vitro fermentation of breast milk oligosaccharides by Bifidobacterium infantis and Lactobacillus gasseri.Applied and Environmental Microbiology. p. 4497-4499.

Watanuki, M., Wada, Y. and Matsumoto, K., (1996). Digestibility and physiological heat of combustions of β-1-6 galactooligosaccharides in vitro. Ann Report Yakult Central Inst Microbiol Res 16, 1–12.

Wichienchot, S., Prasertsan, P., Hongpattarakere, T., Gibson, G. R., and Rastall, R. A., (2003). In vitro fermentation of mixed linkage glucooligosaccharides produced by gluconobacter oxydans NCIMB 4943 by the human colonic microflora. Curr Issues Intestinal Microbiol. 7: 7–12.

Worthley, D. L, Le Leu, R. K., Whitehall, V. L., Conlon M., Christophersen C., Belobrajdic D., Mallitt K. A., Hu Y., Irahara N., Ogino S., Leggett B. A., Young G. P., (2009). A human, double-blind, placebo-controlled, crossover trial of prebiotic, probiotic, and synbiotic supplementation: effects on luminal, inflammatory, epigenetic, and epithelial biomarkers of colorectal cancer. Am J Clin Nutr. 90(3):578-86.

Yanahira, S., Kobayashi, T., Suguri, T., Nakakoshi, M., Miura, S., Ishikawa, H. and Nakajima, I. (1995). Formation of oligosaccharides from lactose by Bacillus circulans β-galactosidase. Biosc Biotech Biochem 59, 1021–1026.

Yang, S. T., and Silva, E. M., (1995). Novel products and new technologies for use of a familiar carbohydrate, milk lactose. J Dairy Sci 78, 2541–2562.

Yang R., Xu S., Wang Z., Yang W., (2005). Aqueous extraction of corncobxylan and production of xylooligosaccharides. LWT Food Sci Technol 38: 677–82.

Yoo, S. H., Kweon, M. R., Kim M. J., Auh, J. H., Jung, D. S., Kim, J. R., Yook, C., Kim, J. W., and Park, K. H., (1995). Branched oligosaccharides concentrated by yeast fermentation and effectiveness as a low sweetness humectant. J Food Sci. 60 (3): 516–519.

Yoshioka, H.; Iseki, K., and Fujita, K., (1983). Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. Pediatrics 72:317-321.

Yun, J., Lee, M., and Song, S. (1994). Continuous production of isomaltooligosaccharides from maltose syrup by immobilized cells of permeabilized Aureobasidium Pullulans. Biotechnol Lett. 16: 1145–1150.

APPENDIX

Fig. 1 Vacuum evaporator.

Fig. 2 Microtiter plate.

Fig. 3 Reflektoquant RQflex10 equipment.

Fig. 1 Vacuum evaporator.

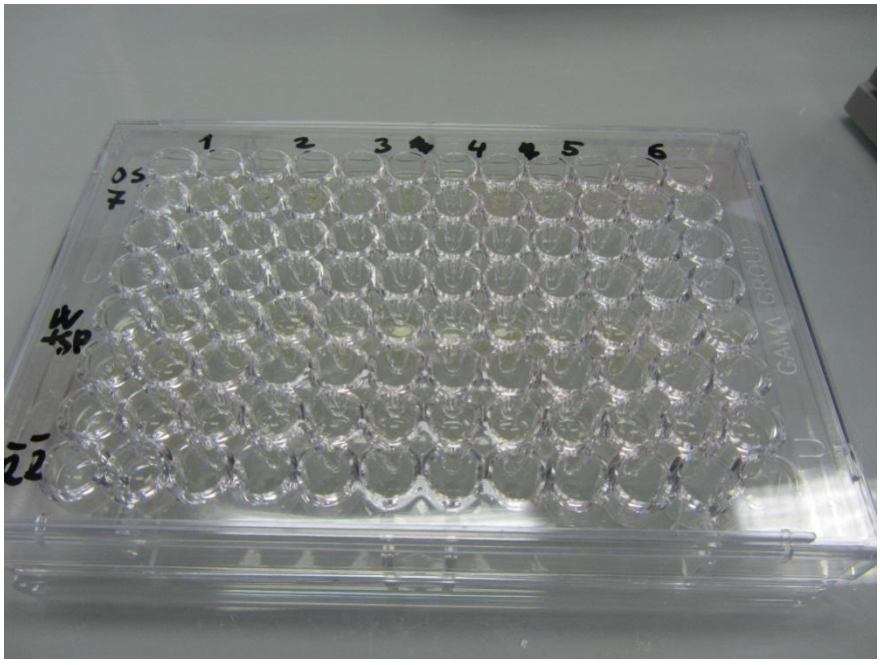


Fig. 2 Microtiter plate.

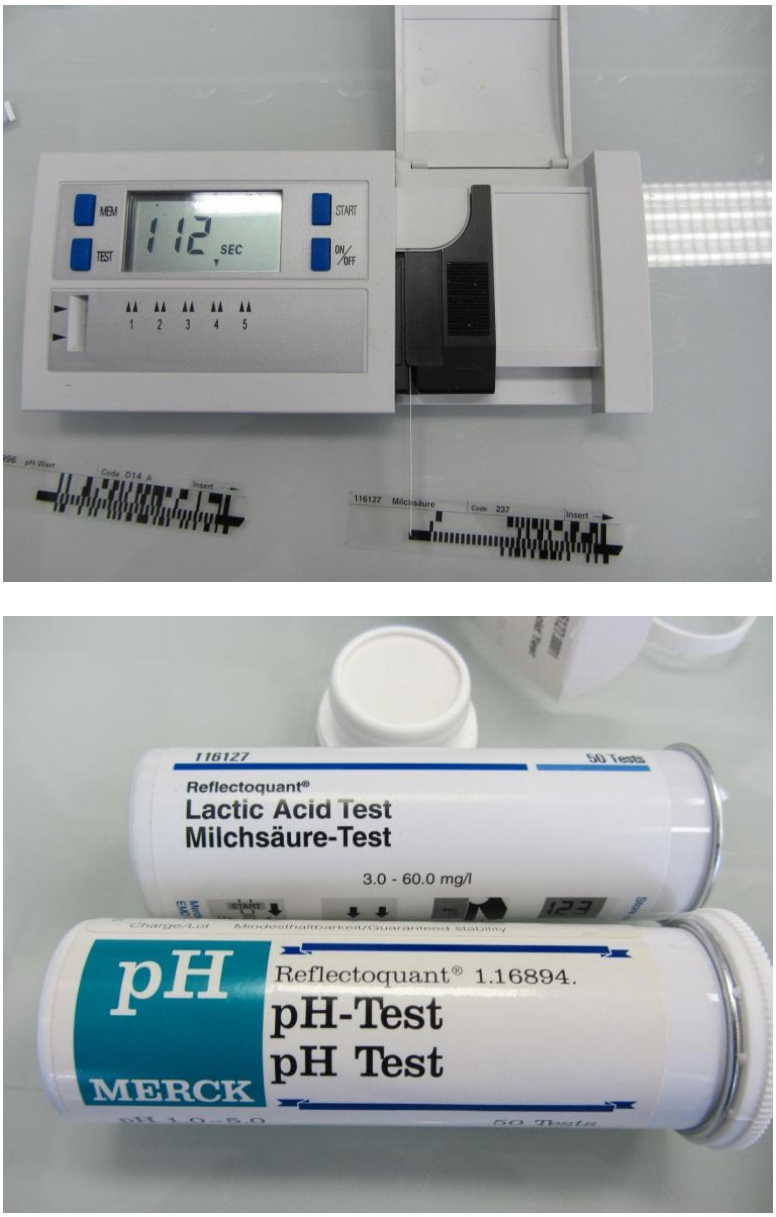


Fig. 3 Reflektoquant RQflex10 equipment.