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5. History, synthesis, properties, applications and regulatory issues of prebiotic oligosaccharides

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Abstract. In this chapter, the health promoting effects of carbohydrate prebiotics are addressed. A brief description of their synthesis, thermo-physical properties, mechanisms of action, technological applications and current regulatory issues are presented.

1. Introduction

Lactobacilli and bifidobacteria are considered as outstanding examples of health promoting constituents of the intestinal microbiota. The main health promoting effects include immunological stimulation, improved digestion and absorption, vitamin synthesis, inhibition of the growth of potential pathogens, cholesterol reduction, lowering of gas distension and restoration the normal flora after antibiotic therapy [T.C. Wallace, 2011].

The development of the intestinal microbiota is controlled and modulated by different interacting mechanisms such as genetic endowment, intrinsic

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biological regulatory functions and environmental constraints. Moreover, the role of the diet can be crucial. This role involves the intake of living organisms and/or the selective stimulation of health promoting bacteria by the intake of certain non-digestible food ingredients, known as prebiotics.

Supplying an exogenous source of live microorganism is not always an easy task considering that not all microorganisms can overcome the passage through the gastro-intestinal tract (GIT) and reach the gut in sufficient amounts to exert a benficial effect. This is especially relevant in the case of bifidobacteria, a genus particularly sensitive to the aggresive environment of the GIT (pH, bile, etc).

For this reason, the modulation of the indigenous intestinal microbiota without an exogenous intake of live bacteria represents an appropriate approach to promote health benefits circumventing the contact of sensitive microorganisms with the aggresive GIT environment. In this context, prebiotics, which are stable enough to withstand such conditions, represent a good option to overcome such limitation.

2. History of prebiotics

The first reference related to the concept of prebiotic dates back from 1954. Gyorgy reported that N-acetyl-glucosamine, a component of human milk promoted the growth of a *Bifidobacterium* strain. In 1957, Petuely recognized lactulose as a *bifidus factor*. Some years later, in the seventies and eighties (XX^{th} century), Japanese researchers reported that several non-digestible oligosaccharides were *bifidus factors*. This opened up a new approach in the study of intestinal microbiota. The term prebiotic was defined for the first time in 1995 [G.R. Gibson and M.B. Roberfroid 1995].

Prebiotics are defined as non-digestible food components that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health. Therefore, the ingestion of food ingredients with well-known prebiotic properties can positively modulate the intestinal microbiota [G.R. Gibson and M.B. Roberfroid 1995].

There are some requirements for a food ingredient to be considered as prebiotic:

a) it must be refractant to hydrolysis and absorption in the upper part of the GIT,
b) it must selectively promote bifidobacteria and/or lactobacilli in the colon, thus being able to modify the intestinal microbiota to a healthier composition,

c) its fermentation products should induce beneficial luminal and/or systemic effects within the host,
d) it must withstand the process conditions at which the food that bears it is subjected.

From a chemical point of view, most of prebiotics and prebiotic candidates identified up to now are non-digestible oligosaccharides (NDOs).

Prebiotics effects have been primarily addressed toward the colon, but there is increasing evidence that prebiotics also exert their effect beyond the GIT [I. Lenoir-Wijnkoop et al. 2007]. In this sense, prebiotics may directly stimulate immunity, protect against pathogens and facilitate host metabolism and mineral absorption [I. Lenoir-Wijnkoop et al. 2007]. Figure 1 schematically represents different prebiotics effects:

Investigation of the genes responsible for fermentation of prebiotics in lactobacilli and bifidobacteria has highlighted the role of specific enzymes and oligosaccharide transporters for degradation of prebiotics [D.M.A.

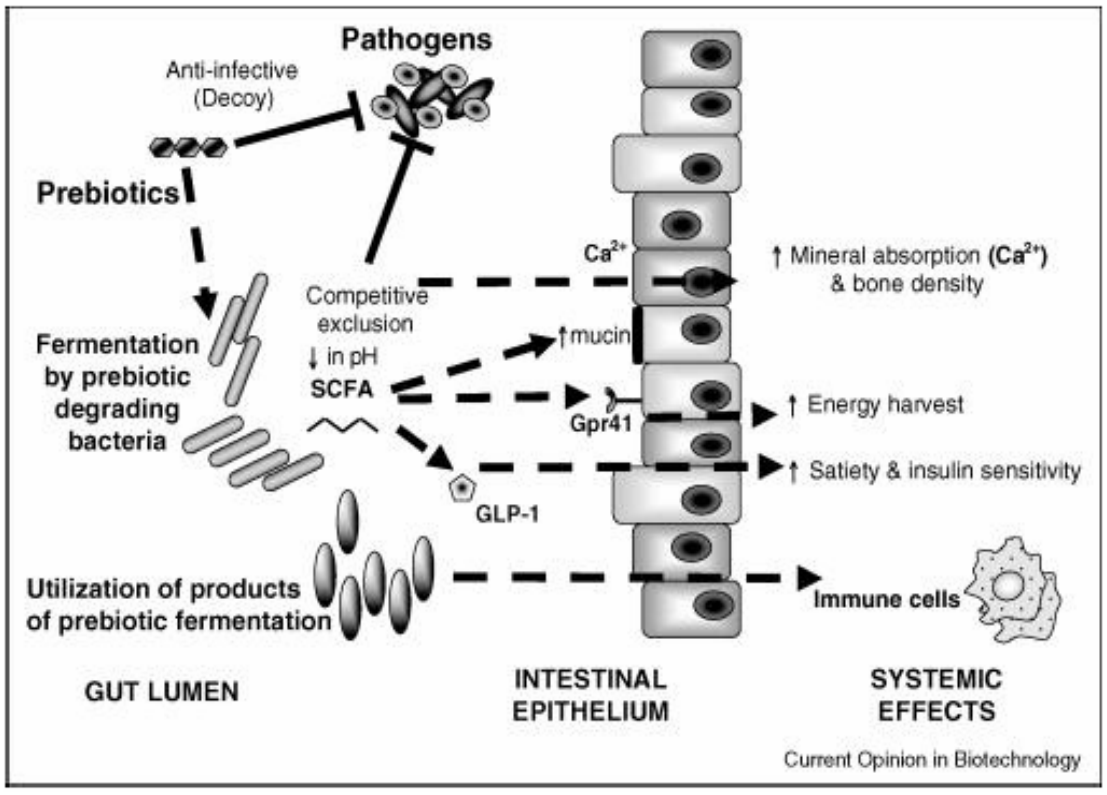

Figure 1. Schematic representation of the beneficial roles of prebiotics in the mammalian GIT and their systemic effects. Ca^{2+}: calcium; GLP-1: glucagon-like peptide-1; Gpr41: G-protein coupled receptor 41; SCFA: short-chain fatty acid. From D.M.A. Saulnier et al. 2009.

Sauhier et al. 2007; R. Barrangou et al. 2006; R. Gonzalez et al. 2008; Y.J. Goh et al. 2007]. Lactic acid and acetic acid, both produced after prebiotic fermentation by lactobacilli and bifidobacteria, can be subsequently degraded by other microorganisms, to give short chain fatty acids (SCFAs), namely propionate and butyrate. These acids are a source of energy for the host and also inhibit the overgrowth of putrefactive bacteria.

Prebiotics can also facilitate the competitive exclusion of potential pathogens and also modulate the immune system, enhancing host defenses. SCFAs are able to improve mucosal morphology by increasing mucin production and decreasing translocation by binding to SCFA receptors on immune cells within the gut lymphoid-associated tissue (GALT) [A.R. Lomax and P.C. Calder 2009]. In particular, butyrate (product of prebiotic fermentation) inhibits the growth of colonic cancer cells *in vitro* [B.L. Pool-Zobel and J. Sauer 2007].

It has been suggested that prebiotics can act as decoy for pathogen-binding cellular receptors in the gut [K.D. Shoaf-Sweeney and R.W. Hutkins 2009]. There exists some evidence suggesting that some prebiotics may have a potential effect on the reduction of the risk of atherosclerotic cardiovascular disease [R. Crittenden and M.J. Playne 2009].

Finally, prebiotics contained in food may also offer further benefits, such as the improvement of minerals absorption (*i.e*. calcium or magnesium) and confer other physicochemical attributes to the food matrix [D.M.A. Saulnier et al. 2009].

Evaluation of the health promoting effect led by prebiotics

The prebiotic effects of oligosaccharides may be influenced by different factors, namely:

-Monosaccharide composition: the building blocks of recognized prebiotics are glucose, galactose, xylose and fructose.

-Glycosidic linkage: it is crucial in determining both selectivity of fermentation and digestibility in the small intestine.

-Molecular weight: The most common prebiotics are oligosaccharides with a relatively small degree of polimerization (DP), the exception being inulin, whose prebiotic effect is less significant than the one of small molecular weight oligosaccharides.

In adults, the intake of prebiotics induces between 10 to 100 fold increase in the size of the intestinal bifidobacteria population during the period of ingestion. However, the daily effective dose of the prebiotic is determined by

the initial size of the intestinal population of bifidobacteria. When it is high [~10^8 cell forming units (CFU)/g of feces], consumption of prebiotics does not further increase their number [R. Crittenden and M.J. Playne 2009]. Moreover, the prebiotic effect of different compounds becomes difficult to compare if no quantitative tools are used.

The accurate description of prebiotic activities requires the definition of a quantitative parameter. This parameter is the prebiotic index (PI), defined as "the increase in the absolute number of bifidobacteria expressed divided by the daily dose of prebiotic ingested. [R. Palframan et al. 2003; M.B. Roberfroid 2007; K. Manderson et al. 2005]. Palframan has proposed a more elaborated prebiotic index that takes into account both the stimulating effect on health promoting bacteria (bifidobacteria and lactobacilli) and the depressing effect on prutefactive bacteria (clostridia and bacteroides).

The following equation describes the PI:

$$PI = Bf_t/Bf_0 - Bac_t/Bac_0 + Lac_t/Lac_0 - Cl_t/Cl_0$$

where Bf_t is the bifidobacteria number after the intake of a given prebiotic, Bf_0 is the initial bifidobacteria number, Bac is the bacteroides number after the intake of a given prebiotic, Bac_0 is the initial bacteroides number, Lact is the lactobacilli number after the intake of a given prebiotic, Lac_0 is the initial lactobacilli number, Cl_t is the clostridia number after the intake of a given prebiotic and Cl_0 is the initial clostridia number.

This equation assumes a positive effect associated to the increase in the populations of bifidobacteria and lactobacilli and a negative effect, related to the increase in bacteroides and clostridia. The expression of bacterial numbers at sampling time in relative terms (referred to the total population at inoculation) allows obtaining an index independent to the variable initial level of each population. In other words, considering these ratios in the PI equation represents a way to normalize the bacterial numbers at sampling times.

Using the PI equation, C.E. Rycroft et al. (2001) compared the fermentation patterns of commercially available prebiotics. The authors found that galacto-oligosaccharide (GOS) and lactulose are those with the greatest prebiotic effects. Isomalto-oligosaccharides and soybean oligosaccharides have also high PI scores. On the other hand, PIs of fructo-oligosaccharides (FOS) and inulin are considerably lower than that of GOS.

Finally, it must be underlined that the PI also represents a useful tool in the development of new prebiotic carbohydrates. Moreover, this quantitative parameter may facilitate a more rational evaluation of health foods and

establish guidelines for the development of more potent prebiotics, active at lower doses.

3. Synthesis of prebiotics

Functional foods market has experienced a substantial development in the last decade with increase rates of 10 to 15 % per year, much higher than food market as a whole whose increase is about 2 % per year. The European market was close to 10 million Euros in 2005 and reached 15 million in 2010 (RTS Resources Ltd). In 2002, the USA market for functional foods was around US$ 20 million with an increase rate of 7 % per year [N.J. Matella et al. 2006]. According to a survey by Frost & Sullivan in 2008 (http.//www. reuters.com/article/pressRelease), the prebiotic market in USA was US$ 70 million and may reach US$ 200 million by 2015, which represents an increase rate of about 15 % per year. Japan was the first country incorporating NDOs in foods and is the world leader in the use of prebiotics. The concept of foods for specific health use (FOSHU) was coined in 1991 in Japan and there is an explosive increase in the number of FOSHU products now in the market. In Japan, the NDO market reached US$ 125 million in 2001, and today about 50 % of FOSHU products contain NDOs [H. Tanigushi, 2005].

In principle, any foodstuff reaching the colon is a potential prebiotic; however, NDOs are those that more closely meet all requirements to be properly considered as such [S.I. Mussatto and I.M. Mancilha 2007]. Beyond their prebiotic nature, NDOs are endowed with interesting properties as food ingredients, namely, non-cariogenicity and low calorific value; they have also been associated with reducing the risks of infection and diarrhea by stimulating the immune response of the host [M. de Vrese and P.R. Marteau, 2007]. Within NDOs, there is a wide variety of compounds that share prebiotic qualifications. They include: inulin [M.B. Roberfroid, 2002], FOS [E. Biedrzycka and M. Bielecka, 2004], xylo-oligosaccharides [Z.Q. Jiang et al. 2004; I. Maalej-Achouri et al. 2009], isomalto-oligosaccharides [T. Nakakuki 2002; C. Moulis et al. 2008], GOS [R.A. Rastall 2006; B. Splechtna et al. 2006] including soybean galacto-oligosaccharides [S. Kim et al. 2003; I. Espinosa-Martos and P. Rupérez 2006], lactulose [Y.S. Kim et al. 2006], lactitol [A. Piva et al. 1996; K.F. Kummel and S. Brokx 2001] and lactosucrose [T. Ohkusa et al. 1995; M.J. Playne and R.G. Crittenden 2004], and some others like gentio-oligosaccharides [S.I. Mussatto and I.M. Mancilha 2007; R.G. Crittenden and M.J. Playne 1996], lactobionic acid [M. Saarela et al. 2003] and tagatose [G. Schaafsma 2008]. A complete summary on the beneficial effects and potential risks of NDOs as prebiotics

has been published [R. Crittenden and M.J. Playne 2009]. Nowadays, inulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides GOS) and lactulose are properly considered as prebiotics, while isomalto-oligosaccharides (IMO) and xylo-oligosaccharides (XOS) do not meet yet all requirements to be considered as such [G.R. Gibson et al. 2004]. A thorough comparative study of NDOs in terms of prebiotic efficacy was conducted by C.E. Rycroft et al. (2001). Most NDOs are produced by synthesis (either chemical or enzymatic). The most relevant in terms of their potential as prebiotics are outlined below.

Fructo-oligosaccharides (FOS)

FOS are small chain oligosaccharides composed by fructose units linked by (2→1)-β-glucosidic bonds and a single D-glucosyl unit at the non-reducing end of the chain. FOS are mostly a mixture of the trisaccharide 1-kestose, the tetrasaccharide, nystose and the pentasaccharide fructosyl nystose [J.M. Campbell et al. 1997]. FOS have been conclusively proven as prebiotic although its prebiotic index is reported as significantly lower than those of GOS or lactulose [C.E. Rycroft et al. 2001].

Industrial production of FOS is two-way. One process is based on inulin hydrolysis, where inulin is water extracted from plants and, after refining, FOS are produced by partial enzymatic hydrolysis with endo-inulinase [A. Franck, 2002]. The other one is based on the enzymatic transfructosylation of sucrose with bacterial or fungal fructosyl transferases [P.T. Sangeetha et al. 2005] or fungal β-fructofuranosidase [Q.D. Nguyen et al. 1999]. Higher molecular weight FOS are produced by the former process, while short chain FOS are produced by the latter. Prebiotic effect is stronger for short chain FOS [E. Biedrzycka and M. Bielecka 2004] so that, at least for the purpose of using the product as prebiotic, enzymatic transfructosylation with fructosyl transferases or β-fructofuranosidases is the technology of choice. Several technologies have been proposed for the enzymatic synthesis of FOS from sucrose, being mostly conventional batch processes with the free enzyme [J.W. Yun 1996] or continuously operated processes with either immobilized enzymes or cells [E. Biedrzycka and M. Bielecka 2004] the ones used industrially. Commercial FOS is a rather impure product containing at least 45 % residual glucose, fructose and sucrose, the rest being 1-kestose, nystose and fructosyl nystose in variable proportions depending on the enzyme source used for the synthesis. A typical product contains 25-30 % (w/w) 1-kestose, 10-15 % (w/w) nystose and 5-10 % (w/w) fructosyl nystose [T. Casci and R.A. Rastall 2006]. Selective removal of monosaccharides from FOS has been accomplished in

packed-bed columns of zeolite [R.C. Kuhn and F.M. Filho 2010] and activated carbon [C. Nobre et al. 2012]. High FOS syrups with purity as high as 98 % can be produced by removing residual glucose using a mixed enzymatic system with beta-fructofuranosidase and glucose oxidase [D.C. Sheu et al. 2001]. Simultaneous removal of glucose, fructose and sucrose has been attempted by treating a commercial FOS preparation with immobilized cells of *Zymomonas mobilis* with significant reduction of those sugars [R.G. Crittenden and M.J. Playne 2002]. Some commercial FOS products are the Japanese Neosugar and the USA product Actilight, both produced enzymatically using enzymes from *Aspergillus niger* [T. Casci and R.A. Rastall 2006]. Design and economics of industrial production of FOS has been reported by K. Vaňková et al. (2008). Beyond its prebiotic condition, FOS have been reported to have anticancerous effect, control of diabetes, reduction of uremia and exert systemic effect in hepatic lipid metabolism, and FOS are used in different food products as non-caloric sweetening agent and in mixtures with inulin to improve organoleptic and functional properties of yoghurts [P.T. Sangeetha et al. 2005]. Strictly as prebiotic, FOS are being used in mixtures with GOS in special milks for infants and the elderly.

Lactose derived-oligosaccharides

Lactose (β-D-galactosyl-D-glucose) is a plentiful material representing most of the carbohydrate portion in milk, which is its only natural source. Lactose and lactose derived products have often been considered merely as ways of overcoming whey disposal problem in cheese-making, being whey a major pollutant because of its high BOD [J.G. Zadow 1984; S.S. Marwaha and J.F. Kennedy 1988]. Whey market is quite unstable and prices go up and down, so an industrial platform for whey utilization is highly desirable. Among the many options of whey (or whey permeate) upgrading, the production of lactose-derived non-digestible oligosaccharides (NDOs) is mostly relevant [A. Illanes 2011]. Lactose is a very rich source for candidate prebiotics like lactitol, lactobionic acid, lactosucrose, tagatose, lactulose and GOS. From the above listed, only GOS and lactulose have conclusively proven their effectiveness and are properly considered as prebiotics [H. Barreteau et al. 2006]. The others, even though not complying with all the requirements to be considered as such are recognized as health promoting NDOs and many of them are also marketed for improving organoleptic and functional properties of foods containing them and in some cases as pharmaceutical products. Most significant

lactose-derived NDOS are surveyed below. Some of them are produced by enzyme catalysis, while others are produced by chemical synthesis

-Lactitol. It is a sugar alcohol (4-β-galactopyranosyl-sorbitol) produced by chemical hydrogenation of lactose [M.G. Gänzle et al. 2008]. Most of lactitol is metabolized to SCFAs by the colonic microbiota [W.L. Dills et al. 1989] and in this sense, it can be considered a prebiotic. Moreover, lactitol has been used as an alternative to lactulose for the treatment of hepatic encephalopathy [B. Als-Nielsen et al. 2004], being also applied as a non-caloric sweetener for diabetics [H. Young 2006].

-Lactobionic acid. It is a sugar acid (4-O-β-galactopyranosyl-D-gluconate) produced by chemical oxidation of lactose, although it can also be synthesized by glucose-fructose oxidoreductase [M. Satory et al. 1997]. Even though it is resistant to digestive enzymes, not absorbable in the small intestine and fermented in the colon, its prebiotic effect has not yet been proven conclusively [M. Saarela et al. 2003]. It is also used as a powerful chelating agent in calcium supplement tablets, as sequestrant in detergents and also in organ preservation for transplants [M.G. Gänzle et al. 2008].

-Lactosucrose. It is a trisaccharide (β-D-fructofuranosyl 4-*O*-β-D-galactopyranosyl-α-D-gluco-pyranoside) derived from lactose by transfructosylation, which is already a commercial product in Japan. Transfructosylation can be accomplished with bacterial or fungal fructosyltransferases [A. Pilgrim et al. 2001; W. Li et al. 2009], or cells containing such activity, using sucrose or raffinose as donor. Even though its bifidogenic effect and inhibitory effect on clostridia is well documented, it is still considered not to fully comply with the requirements of a prebiotic [M.G. Gänzle et al. 2008].

-Tagatose. It is a galactose isomer that can be derived from lactose after hydrolysis and glucose separation using the enzyme arabinose isomerase to convert galactose into tagatose [S.A. Ryu et al. 2003; P. Kim et al. 2001 and 2004]. Tagatose is largely undigested in the small intestine and fermented in the colon being a prebiotic candidate [H. Bertelsen et al. 1999]. It received GRAS status and is being currently used as a healthy low-calorie sweetener [G.V. Levin 2002].

-Lactulose. It is a disaccharide (4-O-β-D-galactopyranosyl-D-fructose) not present in nature, although it is produced by isomerization of lactose during heat treatment of foods containing it [E. Marconi et al. 2003]. It is produced from lactose by chemical isomerization at alkaline conditions [F. Zokaee et al. 2002; M. Aider and D. de Halleux 2007]. However, its enzymatic production from lactose with β-galactosidase using fructose as

galactosyl acceptor has also been explored as an option, having the potential of a more efficient process (higher conversion, less downstream processing less energy consumption) and more environmentally sound (less offensive waste streams to be treated) [C. Guerrero et al. 2011]. Quite recently Y.S. Kim and D.K. Oh (2012) have reported the one substrate synthesis of lactulose from lactose using cellobiose 2-epimerase from *Caldicellulosiruptor saccharolyticus*, which represents a significant step further for implementing the enzymatic process at industrial level. Lactulose outstands among NDOs and has been considered properly as a prebiotic even though its bifidogenic effect is less pronounced that of GOS [M.G. Gänzle et al. 2008]. Beyond its prebiotic condition, lactulose is used as a laxative for the treatment of acute and chronic constipation [Y. Tamura et al. 1993] and for the treatment of hyperammonemia and chronic hepatic encephalopathy [R. Crittenden and M.J. Playne 2009; B. Als-Nielsen et al. 2004]. A thorough review on medicinal properties of lactulose as well as its prebiotic condition has been recently published [R. Schuster-Wolff-Bühring et al. 2010]. Beyond that, lactulose is sweeter and more soluble than lactose which makes it interesting as an ingredient in baking and confectionery [T. Mizota et al, 1987].

-Galacto-oligosaccharides (GOS). They are NDOs composed by a variable number of galactose units (usually from two to ten) and a terminal glucose unit, linked mostly by β1-4 and β1-6 bonds [T. Casci and R.A. Rastall 2006; C. Vera et al. 2011]. Prebiotic effect is mostly associated with the trisaccharide (GOS-3) and tetrasaccharide (GOS-4). Different from other lactose derived prebiotics, GOS are commercially produced by biocatalysis using fungal and bacterial β-galactosidases [M.G. Gänzle et al. 2008], the biocatalytic route having displaced the more cumbersome chemical synthesis [P. Monsan and F. Paul 1995; P. Sears and C.H. Wong 2001]. GOS are already being produced commercially by enzyme technology in Japan and Europe; some of the most relevant companies are Yakult Honsha Co Ltd (www.yakult.co.jp) and Nissin Sugar Manufacturing Co Ltd (wwww.nisin-sugar.co.jp) in Japan, and Friesland Foods in The Netherlands (www.borculodomo.com). β-galactosidases from fungi of the genera *Aspergillus* and yeasts of the genera *Kluyveromyces*, both having GRAS status, are the most adequate for industrial use [A. Illanes et al. 1993], having been traditionally employed in the food [V. Gekas and M. López-Leiva 1985; S.U. Rehman 2009] and pharmaceutical industries [C.J. Booij 1985], even though β-galactosidases from thermophilic and psychrophilic microorganisms [S. Sheik Asraf and P. Gunasekaran 2010], and several probiotic lactobacilli [D. Roy et al.

2002; S. Iqbal et al. 2011] have been also proposed as suitable producing strains. However, it must be recalled that these enzymes are used in its hydrolytic capacity, so the question is how good are they to perform in reverse, catalyzing reactions of transglycosylation. In this context, the β-galactosidases from *Aspergillus oryzae* [N. Albayrak and S.T. Yang 2002; C. Guerrero et al. 2011] and *Bacillus circulans* [P.S. Panesar et al. 2006] are at present the best choice for GOS synthesis. The enzyme from *A. oryzae*, despite producing lower lactose to GOS conversion yields, may be considered a better option due to its low price, excellent operational stability and excellent GOS profile. β-galactosidase immobilization has been intensively studied to improve GOS production [N. Albayrak and S.T. Yang 2002; T. Sakai et al. 2008; L.M. Huerta et al. 2011; D. Sen et al. 2011a]; however, immobilization does not solve the problem of product purity, so synthesis with immobilized β-galactosidase is not for the moment an alternative to the use of the enzyme dissolved in the reaction medium. Besides, being β-galactosidase a commodity enzyme, optimization of biocatalyst use is not critical for process economics, being downstream processing of the product a more critical issue. In fact, commercial GOS products are rather impure with total GOS amounting no more than 50 % of solids, and its composition, both in type of linkage and molecular size distribution, varies significantly according to the origin of the enzyme [R.A. Rastall 2006]. Glucose and residual lactose are the main contaminants that need to be removed to different extents according to the intended use of the product. Current technology for GOS purification is based on chromatographic operations but, being expensive and hard to scale-up [J.I. Sanz Valero 2009; A. Gosling et al. 2010]. Other options like selective fermentation [C.C. Cheng et al. 2006; A. Goulas et al. 2007; Z. Li et al. 2008], membrane fractionation by nanofiltration [A.K. Goulas et al. 2003; Y.M. Feng et al. 2009; V.A. Botelho-Cunha et al. 2010] and *in-situ* product purification by selective removal of contaminating sugars by either adsorption [M.A. Boon et al. 2000; O. Hernández et al. 2010] or precipitation [Sen et al. 2011b] have been proposed.

The prebiotic condition of GOS is beyond any doubt, its beneficial effect being proven consistently in in-vitro [R.J. Palframan et al. 2002] and also in *in-vivo* trials in model animals [Z. Djouzi and C. Andrieux 1997] human adults [Y. Bouhnik et al. 1997] and infants [X.M. Ben et al. 2004], although there is some controversy of the in-vivo effect [M. Alander et al. 2001]. GOS are particularly suited as prebiotic in milk and dairy products: GOS are natural components of human breast milk so its addition to infant formula [F. Savino et al. 2003] and yogurts as a prebiotic or as symbiotic mixture with

probiotics is in practice both in Japan and in Europe [L. Lamoureux et al. 2002; U. Sairanen et al. 2007]. Its use is quite extended in the Japanese food industry where it is included in a variety of products like bread, jams, sport drinks, confectionery and desserts [R.A. Rastall, 2006]. GOS are slightly sweet (about 40 % relative to sucrose) and quite stable, even at high temperatures and low pHs [A.G.J. Voragen 1998]. Beyond their prebiotic condition, GOS are interesting functional food ingredients: they are non-cariogenic and their excellent taste, acid resistance and moderate sweetness make them appealing as functional sweeteners [T. Sako et al. 1999; B. Splechtna et al. 2006].

4. Thermo-physical properties of prebiotics

Investigating thermo-physical properties of prebiotics is important as a background to understand their technological properties. As the most widely used prebiotics are carbohydrate derivatives, the thermo-physical properties of prebiotics are actually, the thermo-physical properties of carbohydrates, which are crucial in food technology. Therefore, insights on physical-chemical properties of prebiotics have relevant implications in the development of new products [B. Higl et al. 2007].

GOS and FOS are available as dried powders that can be found in an amorphous metastable state (glassy state), highly dependent on the temperature and the moisture content. These amorphous sugar powders are highly hygroscopic. The increase of temperature results in plasticization, allowing the material to adopt a more liquid-like *rubbery* amorphous structure. The glass transition temperature (T*g*) is the temperature at which the change from the glassy to the rubbery state takes place. Stability of amorphous products is largely determined by the T*g*, which in turn depends on storage conditions (*i.e.*: water activity, temperature).

The value of T*g* is specific for each carbohydrate or carbohydrate mixture, and it is proportional to its molecular weight [A.K. Shrestha et al. 2007; Y.H. Roos 1993 and 1995]. This relationship has also been reported for glucose homopolymers (maltodextrins) and homopolymers of aldohexoses (GOS) [A.K. Shrestha et al. 2007; D.P.M. Torres et al. 2011].

The main tool to evaluate the relationship between water activity (a_w) and the equilibrium moisture content at a constant temperature are the sorption isotherms. They provide data about the shelf life of a given product. Plasticizers (*i.e.*: water) cause dramatic decreases in Tgs [Y.H. Roos, 1995]. Above Tg, the molecular mobility is greatly increased and many amorphous compounds crystallize. Therefore, high Tgs difficult crystallization and are

desirable for the stabilization of carbohydrate-containing products during storage. As a consequence, carbohydrate prebiotics of high DP have in principle better technological properties.

Some prebiotic carbohydrates have been used with purposes different from their prebiotic effect and this fact represents an added value for products containing these compounds. The following section gives an insight on the use of prebiotics as cryoprotectants.

5. Other applications of prebiotics

The ability of amorpohus sugars to preserve labile biomolecules in dried systems has been recognized for years in food science, pharmacy and medicine [J.H. Crowe et al. 1998].

Freeze-drying has been the method of choice for the storage of both prokaryotic and eukaryotic cells [X.C. Meng et al. 2008; C.A. Morgan et al. 2006; C. Santivarangkna et al. 2007]. However, during this process, the cells are exposed to different kinds of stress due to the decrease in water activity. Oxidation of membrane lipids, damage to proteins, changes in DNA and bacterial cell walls have been proposed to cause cell death during drying and storage. To specifically investigate membrane damages, liposomes are widely used because they are convenient models to reproduce biological membranes.

To avoid damages in the different biological structures, a common procedure is to add carbohydrates to cells and liposomes before the drying process to create a protective amorphous sugar matrix around each biological entity.

The cryoprotectant capacity of trehalose and sucrose is widely known [J.H. Crowe et al. 1994]. These sugars aid in the protection of both membranes and proteins, probably through a combination of glass formation [Crowe J.H. (1998); Crowe, J.H. Leslie, (1994); W.Q. Sun et al. 1996] and direct interaction [J.H. Crowe et al. 1991; 1994; 1998; A.E. Oliver et al. 2001; E.E. Tymczyszyn et al. 2012]. In the last years, oligo and polysaccharides including inulin, FOS and GOS have also demonstrated to be efficient cryoprotectants [C. Schwab et al. 2007; P.S. Panesar et al. 2006; E.E. Tymczyszyn et al. 2011; C. Cacela and D.K. Hincha 2006; R. Wieneke et al. 2007; D.K. Hincha et al. 2008].

Cacela and D.K. Hincha (2006) reported the effect of different families of oligo-saccharides (fructans, malto-oligosaccharides and manno-oligosaccharides) on the preservation of liposomes upon freeze-drying. They found that structural characteristics of the different oligosaccharides and their DP determine the extent to which they are able to interact with and protect membranes during drying. The protection of membranes fusion during drying is also determinant in the stabilization of liposomes by carbohydrates and it

has been reported that vitrification (formation of an amorphous glassy state) during drying prevents the close approach of vesicles necessary for fusion.

The cryoprotectant properties of GOS have been reported recently [E.E. Tymczyszyn et al. 2011]. Commercial GOS preparations containing high proportions of GOS are highly efficient in the protection of lactic acid bacteria during dessication, being DP3 and DP4 the GOS components with the highest cryoprotective capacity [E.E. Tymczyszyn et al. 2011]. Different thermophysical studies support their cryoprotectant properties [D.P.M. Torres et al. 2011; E.E. Tymczyszyn et al. 2012].

For this reason, the role of GOS as cryoprotectants of lactic acid bacteria represents a strong support for the development of new functional foods. Considering the physico-chemical and nutritional properties of GOS, their interaction with probiotics may be useful for the development of commercial synbiotic products, which could be incorporated into different foods (*i.e.*: infant formulas among others).

6. Current regulatory issues on prebiotics

When discussing the scientific substantiation of health claims for prebiotics regulations one is faced with a very ancient concept since diet and health relationship was initially proposed in the fourth century b.c. by Hippocrates. Today, there is significant scientific agreement that diet plays an important role in health.

It is the position of the American Dietetic Association (ADA) that functional foods, including whole foods and fortified, enriched, or enhanced foods, have a potentially beneficial effect on health when consumed as part of a varied diet on a regular basis, at effective levels. The Association supports research to further define the health benefits and risks of individual functional foods and their physiologically active components [ADA 2004].

Although there is no regulatory definition for “functional foods”, they include a wide variety of foods and food components believed to improve overall health and well-being, reduce the risk of specific diseases, or minimize the effects of other health concerns [ADA 2004; W.R. Kapsak et al. 2011]. The term “functional” implies that the food has some identified value leading to health benefits, including reduced risk for disease to the person consuming it. Leaders in the field agree that, despite the absence of a consensus definition, functional foods will continue to have a major impact on the American and international food supply [S. Agarwal et al. 2006]. A random telephone survey of US consumers conducted for the American Dietetic Association supported the notion that a significant percentage of consumers are interested in diet and its potential role in improving health.

General concepts within the regulatory status around the world

In Europe, the role of the Food Safety Agency (EFSA) in the scientific substantiation of health claims is based on Regulation EC (No) 1924/2006 of the European Parliament and about the Council of 20 December 2006 on nutrition and health claims made on foods. For that purpose, EU claim regulation is focused on consumer protection, free/fair trade of goods and promotion of innovation.

Accordingly, the definition of prebiotics implies for EFSA a health benefit which may be possible to document for general population preferably taking also into account several possible subpopulations and, principally, health claims are likely to be substantiated by a cause and effect relationship. Also, EFSA issued a document to provide guidance on their interpretation of what constitutes beneficial effects and acceptable outcome measures [EFSA Guidance, 2011].

According to EFSA, beneficial health effects require scientific assessment of a benefit while function claims relate to the maintenance or improvement of function. Disease risk reduction claims relate to the reduction of a risk factor of a human disease.

Function claims substantiation requires appropriate study of groups of effects (e.g. bowel function/constipation), as well as gastrointestinal discomfort (validated questionnaires, frequency of symptoms) and defense against pathogens (reduction in numbers of specific pathogens, number of gastrointestinal infections).

According to the EFSA technical guidance for the preparation and presentation of the application for authorisation of a health claim under Regulation (EC) No 1924/2006, the nutrition and health claims dossier structure is based on 5 Parts for the Articles 13.5 and 14 claims application.

The EFSA guidance applies to health claims related to the consumption of a food category, a food, or its constituents (including a nutrient or other substances, or a combination of them), hereafter referred to as food/constituent. The purpose of this guidance is to assist applicants in preparing and presenting their applications for authorisation of health claims which fall under Article 14 of the Regulation, *i.e.* reduction of disease risk claims and claims referring to children's development and health. This guidance will be updated at a later stage to cover applications for authorisation of the health claims which fall under Article 18 of the Regulation, *i.e.* applications for inclusion of health claims in the Community list of permitted claims provided for in Article 13(3) which are based on newly developed scientific evidence and/or which include a request for the protection of proprietary data. It is intended that the guidance will be kept

under review and will be amended and updated as appropriate in the light of experience gained from evaluation of health claim applications.

As specified in the EU Regulation, health claims should be substantiated by taking into account the totality of the available scientific data and by weighing the evidence, subject to the specific conditions of use. In particular, the evidence should demonstrate the extent to which:

- the claimed effect of the food/constituent is relevant for human health,
- a cause and effect relationship is established between the consumption of the food/constituent and the claimed effect in humans (such as: the strength, consistency, specificity, dose-response, and biological plausibility of the relationship),
- the quantity of the food/constituent and pattern of consumption required to obtain the claimed effect could reasonably be achieved as part of a balanced diet,
- the specific study group(s) in which the evidence was obtained is representative of the target population for which the claim is intended.

The guidance presents a common format to assist the applicant in the preparation of a well-structured application. This will also help EFSA to deliver its scientific advice in an effective and consistent way. In accordance with the requirements of the Regulation, the application must contain information on the characteristics of the food/constituent for which a health claim is made. Where applicable, this information should contain aspects considered pertinent to the claim, such as the composition, physical and chemical characteristics, manufacturing process, stability, and bioavailability.

In Canada, food health claims are statements in labelling or advertising that link the consumption of a food to health. As elsewhere, false, misleading or deceptive product representations are prohibited.

Health Canada developed a guidance to make the preparation of a health claim submission more efficient while maintaining the standards that ensure the claim is scientifically valid. In order to be accepted as the scientific basis for a new health claim, an existing systematic review must have been prepared according to the guidelines of a regulatory or scientific organization with standards of evidence that are similar to those of Health Canada. Furthermore, the review should be current and directly address the food/health relationship in the proposed claim. In addition to the systematic review, petitioners are required to submit information about the food that will carry the claim, the proposed health effect, how the food/health relationship relates to the general population (*i.e.*, generalizability), and the feasibility of consuming an effective intake of the food in the context of the Canadian diet.

Health claims for prebiotics regulations

Table 1 provides examples of claims and their specific requirements for products considered as prebiotics [CONFS, 1994; ADA, 2002].

The scientific evidences for functional foods and their physiologically active components can be categorized into four distinct areas: (a) clinical trials, (b) animal studies, (c) experimental *in vitro* laboratory studies, and (d) epidemiological studies. Regardless of the research design, a hypothesis-driven approach to the development and evaluation of the efficacy of functional foods has been recognized as paramount to advancing science in this area. Much of the current evidence for functional foods results already from well-designed clinical trials; however, the foundational evidence provided through the other types of scientific investigation is substantial for several of the functional foods and their health-promoting components [M.B. Roberfroid 1998; Y. Bouhnik et al. 1999].

Table 1. Strength of evidence for functional foods currently on the US market [CONFS, 1994; ADA, 2002].

Functional food	Bioactive component	Health benefit	Type of evidence	Strength of evidence	Recommended amount or frequency of intake	Regulatory status
Jerusalem artichoke, onion powder, ripe banana	Prebiotics/ fructo-oligo-saccharides	Blood pressure control; serum cholesterol reduction	Animal studies; clinical trials	Weak	3–10 g/d	Conventional food

A particular category of functional foods includes whole foods that have been associated with reduced risk of disease. For these whole foods, *in-vitro*, *in-vivo*, or epidemiologic research is available to support their health benefits; however, no health claim exists, partially because of the limited or improperly designed clinical trial data or lack of scientific agreement about the strength of the evidence (Table 1). This category includes the following prebiotics:

- NDOs especially fructans, which may potentially provide health benefits for cardiovascular disease, type 2 diabetes, and intestinal infectious diseases [M.B. Roberfroid 1998];
- prebiotic fiber for maintaining a healthy digestive system (60 % *vs* 48 %);
- prebiotic fiber, found, for example, in certain fruits and vegetables and fortified foods, for maintaining a healthy digestive system.

Solid scientific evidence requires unequivocal clarity regarding the criteria (from study design through wording of the claim) for a dossier

suitable for a positive regulatory opinion. One unintended consequence of the current review process may well be that the responsible companies studying the physiological effects of their probiotic or prebiotic products will decide that continued investment into this line of research is not cost-effective if, in the end, evidence supporting product benefits deemed valid by the scientific community.

Evaluation of evidence to support claims is not a simple process. The Nutrition and Allergies (NDA) scientists must implement challenging legislation and assess a flood of dossiers providing evidence, which in the nature of all research could always be improved. But the process is difficult for industry scientists too, who must prepare a dossier in support of a claim with only general guidance from the NDA. A successful dossier requires not only compelling studies on efficacy, but also specification of a physiological effect that will be considered by the NDA as beneficial and a claim that is worded to accurately reflect the science but also be in compliance with regulations.

EFSA issued a positive scientific opinion on the nutritional ingredient lactulose, marketed by Solvay France under the name Solactis. Solactis® approach, and particularly "galactofructose", an unexpected prebiotic ingredient, which after recognition by the Korean Food and Drug Administration (KFDA) for its bifidogenic properties in South Korea, received a positive opinion from the European EFSA on digestive health and transit regulation.

This food ingredient is now recognized for its beneficial effect on reducing intestinal transit time, with a daily dose of 10 grams of lactulose in a portion of food. EFSA confirmed a direct cause-effect relationship between the consumption of lactulose and a return to normal transit time. Solactis in powder format is produced in France. It won the 2009 Frost & Sullivan "Digestive Health Ingredient of the Year" prize.

Lactulose is a well established laxative. The details concerning Solactis may be helpful for the understanding of the rational beyond EFSA decision. The opinion addresses the scientific substantiation of health claims in relation to partially hydrolysed guar gum and decreasing potentially pathogenic gastro-intestinal microorganisms, changes in SCFA production and/or pH in the gastro-intestinal tract, changes in bowel function, and reduction of gastro-intestinal discomfort.

A harmonized future direction of research, regulation and goals is envisaged for prebiotics. The legislation worldwide assumes that health claims should only be authorised for use after a scientific assessment of the highest generally accepted possible standard. Generally accepted scientific evidence is a well-established concept and is the basis for the peer review process of scientific journals, evaluation of grant applications or scientific

productivity of researchers, and grading recommendations in evidence based medicine [F. Guarner et al. 2011].

One intended consequence of the current review process should be that the responsible companies studying the physiological effects of their probiotic or prebiotic products will decide that continued investment into this line of research is actually cost-effective, with evidence supporting product benefits deemed valid by the scientific community to be communicated to the consumer.

Regulatory authorities shall bring together independent academic and industrial scientists involved in research on fundamental and applied aspects of prebiotics, to forward its mission of fostering high-quality research and communication to society in the fields of prebiotics.

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