# DEGRADATION OF STRUCTURALLY DIFFERENT NON-DIGESTIBLE OLIGOSACCHARIDES BY INTESTINAL BACTERIA:

Glycosylhydrolases of *Bifidobacterium adolescentis*

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# DEGRADATION OF STRUCTURALLY DIFFERENT

# NON-DIGESTIBLE OLIGOSACCHARIDES

## BY INTESTINAL BACTERIA:

## Glycosylhydrolases of *Bifidobacterium adolescentis*

Afbraak van in structuur verschillende
niet-verteerbare oligosacchariden
door darmbacteriën

Glycosylhydrolasen van *Bifidobacterium adolescentis*

## Katrien Van Laere

## Stellingen

1. Oligosacchariden worden niet selectief door bifidobacteriën gefermenteerd (dit proefschrift).

2. Het beschikken over goed gekarakteriseerde substraten leidt tot de ontdekking van nieuwe enzymen (dit proefschrift).

3. OD en pH-metingen geven, in tegenstelling tot HPAEC-analyses, geen informatie over de mate van afbraak en afbraak route van oligosacchariden door darmbacteriën (dit proefschrift).

4. De toepassingsmogelijkheden van nieuwe structureel verschillende oligosacchariden zullen afhangen van adequate *in vitro* bio-essays.

5. Fysiologisch gezien zouden alle niet-verteerbare oligosacchariden als voedingsvezel beschouwd moeten worden. Prosky L (1999). Inulin and oligofructose are part of the dietary fiber complex. J AOAC Int. 82: 223-226

6. Bij het ontwikkelen van gezondheidsbevorderende voeding dient meer aandacht besteed te worden aan de activiteit van de darmflora i.p.v. de samenstelling ervan.

7. De kwaliteit van het leven gaat hoestend en proestend achteruit.

8. Computergebruik leidt naast RSI (Repetitive Strain Injuries) ook tot RSE (Repetitive Spelling Errors).

9. Als de Nederlandse PTT-telecompetitie representatief is voor het EK 2000, dan is België, evenals de door een Belg getrainde Nederlandse Kampioen PSV, de absolute favoriet.

Stellingen behorende bij het proefschrift
Degradation of structurally different non-digestible oligosaccharides
by intestinal bacteria:
Glycosylhydrolases of *Bi. adolescentis*

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ABSTRACT

## VOORWOORD

Yes, 't is af en ik die dacht dat er nooit een einde aan zou komen. Maar het is nu zover en ik kijk met heel veel plezier terug op de tijd die ik bij de vakgroep levensmiddelenchemie gewerkt heb. Ik kan best zeggen dat ik tijdens die periode heel veel geleerd en beleefd heb. Dankzij mijn stage, ook bij LMC, was ik al een beetje gewoon aan de Nederlandse mentaliteit en heb dan ook makkelijk mijn draai gevonden. Dat was natuurlijk niet mogelijk geweest zonder alle mensen die op welke manier dan ook een bijdrage hebben geleverd aan mijn promotieonderzoek. Ik wil dan ook van de gelegenheid gebruik maken om een aantal van mensen extra in de bloemetjes te zetten.

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## ABSTRACT

Non-digestible oligosaccharides (NDOs) are oligosaccharides, which resist digestion in the upper gastrointestinal tract, and which are fermented in the colon by intestinal bacteria. Some NDOs are considered bifidogenic, meaning that they selectively stimulate the growth of bifidobacteria in the colon microbiota. The degradative fermentation of structurally different oligosaccharides by intestinal bacteria was studied in this thesis, in order to establish the potentially bifidogenic effects of various types of NDOs. Structurally different oligosaccharides were produced using different routes. Arabino-, (arabino-)galacto-, (arabino-)xylo-, galacturono-, and rhamnogalacturono- oligosaccharides were derived by enzymatic hydrolysis of plant polysaccharides. By transglycosylation reactions using glycosidases, transgalactooligosaccharides of the α- and ß-glycosyl linkage type were obtained.

The chemical structure of the oligosaccharides clearly influenced their fermentation behaviour. It was concluded that species belonging to different groups, and not only bifidobacteria, have the capability of hydrolysing these oligosaccharides. *Bi. adolescentis*, being a major bifidobacterial species of the adult intestinal microflora, was able to utilise a wide range of oligosaccharides showing its wide range of glycosidases. Two novel arabinoxylan degrading enzymes were purified from *Bi. adolescentis*. These enzymes in combination with a ß-xylosidase are involved in the complete degradation of arabinoxylooligosaccharides. For the utilisation of α-galactooligosaccharides *Bi. adolescentis* produced an α-galactosidase. This α-galactosidase was characterised as a retaining glycosidase and was used for the production of new types of α-galactooligosaccharides. These α-1→6 linked-galactooligosaccharides could be utilised by various strains belonging to bifidobacteria and lactobacilli. Upon growth of *Bi. adolescentis* on transgalactooligosaccharides (TOS) a novel ß-galactosidase was produced, involved in the degradation of TOS. It was speculated that this enzyme was membrane or cell wall associated. After growth of *Bi. adolescentis* on TOS or on hydrolysed arabinogalactan the ß-galactosidase production of *Bi. adolescentis* increased compared to growth on galactose. This increased ß-galactosidase activity could be linked to increased activity towards both polymeric and oligomeric galactan. *In vivo* dietary intervention with TOS also resulted in increased levels of ß-galactosidase activity in feces. Although the nature and specificity of the ß-galactosidase is not yet known it can be concluded that glycosidase activity of the intestinal bacteria might be a useful biomarker of the colonic metabolic activity.

# 1

## General introduction

**INTRODUCTION**

Now a days much attention is paid to the possible physiological effects of oligosaccharides in the gastrointestinal tract. The human gastrointestinal tract constitutes a complex microbial ecosystem comprising several hundred different species of bacteria. The colon in particular is densely populated with in excess of $10^{11}$ bacteria per gram of contents. The human host is not inert to these organisms and their metabolic activities and both can have positive and negative impacts on health. Within the intestinal microflora some bacteria are believed to be beneficial to the host while others are potentially pathogenic. The balance of this ecosystem is dynamic and the maintenance of a community of bacteria, which contains a predominance of beneficial species, is believed to be important in maintaining health. To increase the number of health promoting bacteria in the GI-tract, two separate approaches exist. The first is the oral administration of live beneficial microbes, termed probiotics (1,2). The second strategy is to increase the number of beneficial bacteria already present by supplying them with selective carbon and energy sources that provide them with a competitive advantage over other bacteria in the colon. These selective dietary components were named prebiotics. Increasingly, probiotics and prebiotics are used in combination and this is called synbiotics (3).

A range of non-digestible oligosaccharides (NDOs) has been developed which seem to have the potential to increase bifidobacteria in the colon. It is claimed that a high number of bifidobacteria is beneficial for the host's health. A large amount of bifidobacteria may prevent colonisation of pathogens, and may have positive effects on intestinal peristalsis, the immune system, cancer prevention, cholesterol metabolism and carbohydrate metabolism in the colon (4). Bifidogenic effects of lactulose (5); fructooligosaccharides (6,7); galactooligosaccharides (8,9); raffinose (10); lactosucrose (11,12); isomaltooligosaccharides (13) and xylooligosaccharides (14) have been reported in healthy volunteers and patients. Because these oligosaccharides are non-digestible food ingredients and may potentially benefit the host's health by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, they are defined as prebiotics.

Carbohydrates are usually classified according to their molecular size into sugars, oligosaccharides, and polysaccharides. Such a chemical classification, however, needs to be modified to include physiological effects (15). An important classification from the point of view physiology is digestibility in the small intestine. Various types of carbohydrates are undigestible in the human small intestine: non-starch polysaccharides, resistant starch and non-digestible oligosaccharides (NDOs).

NDOs are defined as those carbohydrates with a low degree of polymerisation (ranging from approximately 2-20 monosaccharide units) which are not digested by the host digestive system. The chemical structures of oligosaccharides may vary widely and are determined by the identity of the monomeric sugar units, the degree of polymerisation, the type of linkage between the monomeric units, the complexity of the molecule (branched or linear) and possible linkage to non-carbohydrates. The physiological effects of carbohydrates have

received little attention. Due to the extraordinary complexity of their structure and subsequently their analysis, interest in oligosaccharides and carbohydrates has been generally limited. In addition, until the late 1960s, carbohydrates were thought to serve only as energy sources and as structural materials.

The ability to function as a prebiotic has become a marketing edge for these products and has promoted research into the ability of oligosaccharides to induce beneficial changes in the composition and metabolism of colonic microflora. This is mainly of importance when the ecological balance of intestinal bacteria is disturbed. Several factors such as stress, the diet and antibiotic treatment can cause such a disturbance, which may result in several acute or chronic diseases. Supplementation with oligosaccharides is a promising tool for prevention of disturbances of the ecological balance.

## OCCURRENCE IN FOOD

Oligosaccharides are present in various natural food products (table 1). They may also occur as glycoconjugates as part of glycoproteins or glycolipids. Fructooligosaccharides are present in various plants belonging to the *Compositae* and *Amaryllidadeae* families (16). α-Galactooligosaccharides are mainly present in legumes and vary in their distribution among leguminous species (17). Their quantities can differ among varieties of a given legume species. The α-galactooligosaccharide content in *Lupinus* species ranges from just below 10% up to 23% of dry matter (18). Many structurally different oligosaccharides have been identified in honey and their composition depended on the type of honey (19). Also, human milk contains significant amounts of various oligosaccharides (20). Non-digestible oligosaccharides may also be formed during food processing due to *hydrolysis* of polysaccharides. Different types of oligosaccharides can be formed in this way during beer brewing (21), the production of wine (22) and bread making (23) and in fruit juices. They *may* also be formed in the large bowel by intestinal bacteria during fermentation of non-starch polysaccharides. Examples of the chemical structure of some naturally occurring oligosaccharides are given in table 1.

**Table 1.** Examples of oligosaccharides which occur naturally in food and feed

| Name | Structure | Origin |
|---|---|---|
| Fructooligosaccharides | (ß-D-Fru$f$-(2→1))$_n$-ß-D-Fru$f$-(2↔1)-α-D-Glc$p$ <br> (ß-D-Fru$f$-(2→1))$_n$-D-Fru$f$ | *Compositae* and *Amaryllidadeae* |
| α-Galactooligosaccharides | (α-D-Gal$p$-(1→6))$_n$-α-D-Glc$p$(1↔2)-ß-D-Fru | Legumes |
| Threanderose <br> Panose | α-D-Glc$p$-(1→6)-α-D-Glc$p$(1↔2)-ß-D-Fru$f$ <br> α-D-Glc$p$-(1→6)-D-α-Glc$p$-(1→4)-D-Glc$p$ | Honey |
| Isomaltooligosaccharides | (α-D-Glc$p$-(1→6))$_n$-D-Glc$p$ | Beer |
| Sialiated oligosaccharides | e.g. α-D-Neu5Ac(2→3)-ß-D-Gal$p$-(1→4)-D-Glc$p$ | Human milk |
| Fucosylated oligosaccharides | e.g. α-Fuc-(1→3)-ß-D-Gal$p$-(1→4)-D-Glc$p$ | Human milk |

## PRODUCTION METHODS

Oligosaccharides are produced commercially using enzymatic processes involving either the hydrolysis of polysaccharides or synthesis starting from smaller carbohydrates using a transglycosylation reaction. Chemical methods may also be used for the synthesis of oligosaccharides. However, carbohydrates contain multiple hydroxyl groups having similar reactivity and the many protection and deprotection steps that are necessary for regioselective synthesis makes such methods very complicated. Currently there are nine different types of NDO's commercially produced, predominantly in Japan and Europe (24).

### Extraction

Since oligosaccharides are naturally occurring substances, they can be extracted from different sources. One example is soybean oligosaccharides (raffinose and stachyose) which are being commercially produced by direct extraction from soy flour. These oligosaccharides are generally considered food-grade and can be added to different food products. Bovine milk contains low levels of the oligosaccharide 3' siallyllactose, which can be extracted from the milk. Since the yield is low, the price of the product is high. New methods are being developed to enrich bovine milk with human milk oligosaccharides. One potential approach is the use of transgenic animals, more particularly transgenic cows that express genes or cDNA encoding enzymes which catalyse the formation of human milk oligosaccharides. These oligosaccharides can then be extracted from the enriched bovine milk (25).

### Transferase reactions

For the preparation of oligosaccharides two types of enzymes have been used: the glycosyltransferases (EC 2.4) and the glycosidases (EC 3.2). For glycosidases this acceptor is water, the result being hydrolysis. For transferases this acceptor is typically an alcohol functionality from another sugar, but it could be from a lipid, aryl moiety, or a range of other components of glycoconjugates. The term glycosyltransferases is generally reserved for enzymes catalysing glycosyl transfer to an acceptor other than water (26). They are classified according to the sugar transferred from donor to acceptor and by the acceptor specificity. The regio- and stereospecificity for the acceptor molecule and the high yields that can be achieved are attractive features of these catalysts.

Glycosidases can be separated into two distinct mechanistic classes: those hydrolysing the glycosidic bond with net inversion of anomeric configuration, and those doing so with net retention (27). The two mechanisms differ in that inverting glycosidases operate via a direct displacement of the leaving group by water, whereas retaining glycosidases utilise a double-displacement mechanism involving a glycosyl-enzyme intermediate. The retaining glycosidases can transfer the glycosyl moiety of a substrate to acceptors other than water and

hydrolysis represents merely a special case in which water serves as an acceptor. Glycosidases show a less pronounced selectivity than the glycosyltransferases for the acceptor molecule. This means that a given glycosidase can be used for the synthesis of a number of glycosides from a given glycosyl donor by employing different acceptors. The less pronounced regioselectivity can result in mixtures of oligosaccharides having different linkages. However, formation of one predominant linkage usually occurs and different linkages may be obtained by using enzymes from different sources (27). The glycosidases occur widely in nature and readily available substrates can be used for the production of oligosaccharides. Generally it can be said that glycosidases are suitable for the synthesis of shorter oligosaccharides while glycosyltransferases are necessary for the synthesis of higher and complex oligosaccharides.

Fructooligosaccharides can be prepared from sucrose though the transfructosylation action of two enzymes, namely ß-fructofuranosidase (3.2.1.26) and ß-D-fructosyltransferase (EC 2.4.1.9) (28) (Fig 1). The oligosaccharides formed are structurally similar to several naturally occurring oligosaccharides (e.g. from onion) (29).
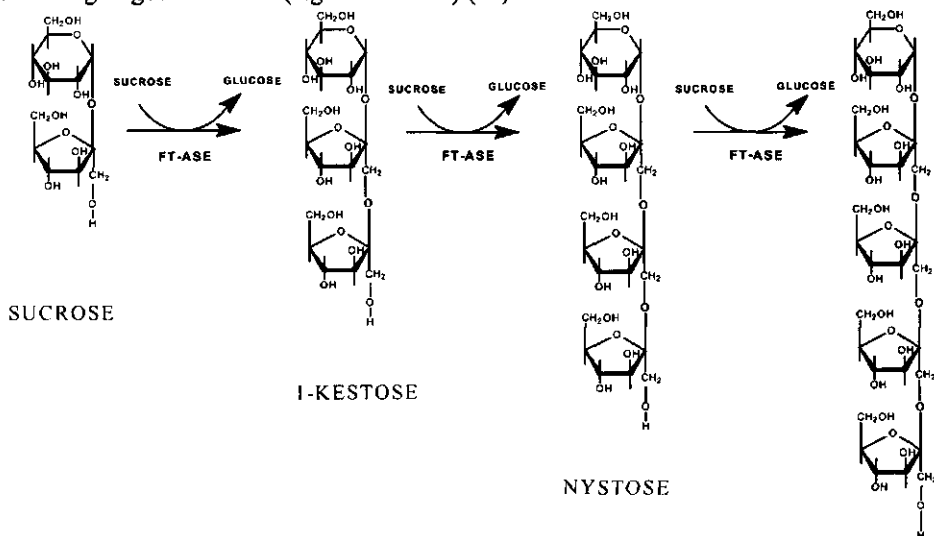


**Figure 1.** Production of FOS by transfructosylation of sucrose using a ß-D-fructosyltranferase (FT-ase)

Transgalactooligosaccharides are made by transgalactosylation of lactose using a ß-galactosidase (30, 31, 32, 33, 34, 35). Some of these oligosaccharides are found in human milk (36) and are present in lactose hydrolysates (37). The amount and nature of the oligosaccharides formed depends upon several factors including the enzyme source, concentration and nature of the substrate, the degree of conversion of the substrate and the reaction conditions. Also lactosucrose, α-glucooligosaccharides and gentiooligosaccharides (38) are produced by transglycosylation reaction. In table 2 examples of commercial available oligosaccharides produced by transglycosylation reactions are given

**Table 2.** Some examples of commercial oligosaccharides produced by transglycosylation reaction

| Group Name | Structure | Sources | enzyme |
|---|---|---|---|
| Fructooligosaccharides | (β-D-Fruf-(2→1))ₙ-(β-D-Fruf-(2↔1)-α-D-Glcp | Sucrose | β-fructofuranosidase |
| Galactooligosaccharides | β-D-Galp-(1→x)-D-Galp | Lactose | β-galactosidase |
| | β-D-Galp-(1→y)-D-Glcp | | |
| | β-D-Galp-(1→4)-β-D-Galp-(1→y)-D-Glcp | | |
| | β-D-Galp-(1→6)-β-D-Galp-(1→x)-D-Glcp | | |
| | β-D-Galp-(1→3)-β-D-Galp-(1→4)-D-Glcp | | |
| | β-D-Galp-(1→z)-[β-D-Galp-(1→6)-D-Glcp] | | |
| | β-D-Galp-(1→4)-[β-D-Galp-(1→2)-D-Glcp] | | |
| | β-D-Galp-(1→3)-β-D-Galp-(1→6)-β-D-Galp-(1→4)-D-Glcp | | |
| | β-D-Galp-(1→6)-β-D-Galp-(1→3)-β-D-Galp-(1→4)-D-Glcp | | |
| | β-D-Galp-(1→6)-β-D-Galp-(1→6)-β-D-Galp-(1→4)-D-Glcp | | |
| | β-D-Galp-(1→6)-β-D-Galp-(1→6)-β-D-Galp-(1→6)-β-D-Galp-(1→4)-D-Glcp | | |
| | (x=4,6; y=2,3,4,6; z=2,3,4) | | |
| Lactosucrose | β-D-Galp-(1→4)-α-D-Glcp-(1↔2)-β-D-Fruf | Lactose, sucrose | β-fructofuranosidase |
| Glucooligosaccharides | α-D-Glcp-(1→x)-α-D-Glcp-(1↔2)-β-D-Fruf | Sucrose, maltose | α-glucosidase |
| Gentiooligosaccharides | (β-D-Glcp-(1→6))ₙ-D-Glcp | Glucose | β-glucosidase |

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**Polysaccharides as source for oligosaccharides**

Plant polysaccharides are a source of carbohydrates containing a variety of different sugar building blocks, linkages and branches and can be used for the production of oligosaccharides. Directed enzymatic hydrolyses can lead to a partial degradation of the plant polysaccharides resulting in oligomers of various degrees of polymerisation and branching. Inulinooligosaccharides (fructooligosaccharides) (39, 40, 41, 42) and xylooligosaccharides (43, 44) are already commercially produced by enzymatic hydrolysis of respectively inulin and xylan. In table 3 the chemical structure of some oligosaccharides produced by hydrolysis of polysaccharides is given.

**Table 3.** Commercially available oligosaccharides produced by hydrolysis of polysaccharides

| Name | Structure | Source | Enzyme |
|---|---|---|---|
| Fructooligosaccharides | (ß-D-Fruf-(2→1))$_n$-ß-D-Fruf-(2↔1)-α-D-Glc$p$ (ß-D-Fruf-(2→1))$_n$-D-Fruf | Inulin | Inulinase |
| Xylooligosaccharides | [ß-D-Xyl$p$-(1→4)]$_n$-D-Xyl$p$ | Xylan | Xylanase |
| Cellodextrins | [ß-D-Glc$p$-(1→6)]$_n$-D-Glc$p$ | Cellulose | Cellulase |

In the same way, other polysaccharides might be valuable sources for the production of (non-digestible) oligosaccharides. Using specific endo-glycanases and glycosidases, various (soluble) fibres can be degraded to a selected degree resulting in a directed production of oligosaccharides (45). The six main polysaccharides commonly found in plant cell walls which are (potential) sources for the production of oligosaccharides are shown in Table 4.

**Table 4.** Polysaccharides commonly found in primary cell walls of higher plants (46)

| Polysaccharide | Structure |
|---|---|
| Cellulose | ß-D-(1→4)-glc$p$ backbone |
| Xyloglucan | ß-D-(1→4)-glc$p$ backbone with xylose, galactose, arabinose or fucose containing side-chains |
| Arabinoxylan | ß-D-(1→4)-xyl$p$ backbone with arabinose, xylose, or glucuronic acid side-groups |
| Rhamnogalacturonan I | →2)-α-L-rha-(1→4)-α-D-galA$p$-(1→ type I arabinogalactan type II arabinogalactan arabinan, galactan side chains |
| Rhamnogalacturonan II | complex low molecular weight (4.8 kDa) segment of pectc polysaccharides (may contain up to 11 different sugars with more than 20 different linkages) |
| Homogalacturonan | α-D-galA$p$-(1→4) backbone |

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## Combination of hydrolysis and transferase reactions

Polysaccharides can also be hydrolysed more extensively using endo-glycanases resulting in the formation of di- and trimeric material which can be used as substrates for further transfer reactions. Isomaltooligosaccharides are glucosyl saccharides with α-(1→6)-glucosidic linkages such as isomaltose, panose and isomaltotriose. Isomaltooligosaccharides are produced from starch via two enzymatic steps. The first step is the hydrolysis of starch by α-amylase, pullulanase and ß-amylase to yield maltose. The second step is the transglucosylation of the glucose moiety of maltose by α-D-glucosidase to produce α-(1→6)-oligosaccharides (47, 48).

## ANALYSIS AND IDENTIFICATION OF CHEMICAL STRUCTURE OF OLIGOSACCHARIDES

Dietary fibre (49) - polysaccharides and lignin included -are not hydrolysed by the endogenous secretion of the human digestive tract (50). Dietary fibre is mainly composed of plant cell wall polysaccharides (cellulose, hemicellulose, pectic substances) and other non-digestible polysaccharides such as intracellular storage polysaccharides (e.g., galactomannans, inulin) and resistant starch. Oligosaccharides are usually excluded in the analysis of dietary fibre and non-starch polysaccharides, since they are soluble in the 80 % ethanol used to precipitate the dietary fibre. However, non-digestible oligosaccharides may reach the large bowel where they can be fermented by the intestinal microflora. Therefore, most professionals in the field recognise NDOs as dietary fibre (51) even though (except for fructooligosaccharides (52)) oligosaccharides are not determined in official AOAC-analysis of total dietary fibre (53).

## Analysis

The analysis of non-digestible oligosaccharides is rather complicated and not straightforward since monosaccharides, dimers like sucrose, maltose, lactose and also maltooligosaccharides should not be included in the analysis.

Various methods have been described for the analysis of oligosaccharides and they are mainly based on separation of the oligosaccharides by HPLC or by GLC after derivatisation (54). The first major breakthrough for the HPLC-analysis of carbohydrates involved the separation of oligosaccharides on an amino-bonded silica column using acetonitrile and water mixtures as eluent. Although this type of column is still quite popular for the analysis of sugars in food products, high-performance anion-exchange chromatography (HPAEC) is also a useful method for the separation of monomeric and oligomeric sugars using high-pH eluents in combination with pulsed amperometric detection. HPAEC permits good separation of structurally closely related oligosaccharides. The monomeric composition, size and linkage

type of oligosaccharides clearly influences the elution behaviour of the oligosaccharides (55, 56, 57, 58), although not always in a predictable way. The high resolution obtained by HPAEC for the separation of complex mixtures of oligosaccharides is accompanied by a high degree of flexibility of the system, which enables adjustment of the gradient for specific applications.

A disadvantage of the system is the lack in uniform response of the PAD detector to the same functional group of carbohydrates. Combining HPAEC-PAD and HPAEC-permanganate post-column detection could solve this problem (59) as it would give similar signals for equal amounts of oligosaccharides and allow quantification of the oligosaccharides. However, this method seems to have some disadvantages because permanganate has the ability to oxidise a wide range of organic compounds. In addition, the permanganate detector is not compatible with several of the commonly used buffers.

A possible alternative might be the initial degradation of the digestible material using a combination of lipases, proteases, amylases and brush-border enzymes, followed by a separation of the oligosaccharides which are obtained using a gelfiltration or amino-bonded silica column in combination with refractive index-detection. RI-measurements are linear over a wide range of carbohydrate concentrations and can be universally applied to all carbohydrates. A serious drawback is the fact that RI-detection is non-specific and non-carbohydrate compounds may therefore co-elute with carbohydrates, causing inaccurate results. However, this combination is probably the best one available because it permits quantification of various types of non-digestible oligosaccharides in contrast to the adapted AOAC-method, which only allows detection of fructooligosaccharides. At the end of the research project, a new detector, which avoids these problems, became available.

## Identification of the chemical structure of oligosaccharides

Not only the quantification of oligosaccharides is important. Determination of their exact structure is essential for establishing their structure-function relationships. To obtain more information on the structure of isolated oligosaccharides or oligosaccharides present in a mixture, one can use various methods and combine the results to determine the corresponding structures.

HPAEC seems to be a versatile method in a growing number of applications, but the identification of unknown compounds using this system is still impossible because of the unpredictable behaviour in the system. An important step forward in the characterisation of unknown components eluting under HPAEC conditions is the on-line combination with mass spectrometry using a thermospray interface (60). Using multiple-ion detection, information on the sugar composition of the oligosaccharides can be obtained. The use of specific enzymes is also an important tool in gaining information on the oligosaccharide structure (61). In addition, NMR-spectrometry may be used to identify unknown oligosaccharides.

## EFFECT OF OLIGOSACCHARIDES ON MICROBIAL BALANCE

### Composition of the intestinal microflora

The colon contains an extremely complex ecosystem in which individual bacteria exist in a multiplicity of different microhabitats and metabolic niches. The microbiota consists of several hundred different bacterial species, subspecies and biotypes. Some organisms occur in higher numbers than others, and about 40 species make up approximately 99% of all isolates (62). The majority of bacterial species in the colon is saccharolytic and can contribute to carbohydrate fermentation. The dominant saccharolytic organisms belong to the genera *Bacteroides*, *Ruminococcus*, *Bifidobacterium*, *Eubacterium* and *Clostridium*. The principle role of the intestinal microflora is to salvage energy from carbohydrates not digested in the upper gut. The major substrates for fermentation are dietary carbohydrates that have escaped digestion in the upper gastrointestinal tract. These include resistant starch as well as non-starch polysaccharides such as cellulose, hemicellulose, pectins and gums. Other carbohydrate sources available for fermentation are mucins, non-digestible oligosaccharides and various sugars and sugar alcohols. Total carbohydrate availability in the human adult colon is 20-60 g carbohydrate/day (63). Significant regional differences occur in bacterial activity in the colon. The right (proximal) colon is characterised by high substrate availability, low pH and rapid transit. The left, or distal, colon has a lower concentration of available substrate, the pH is approximately neutral and bacteria grow more slowly. The proximal colon tends to be a more saccharolytic environment than the distal gut, the latter having higher bacterial proteolysis. Bacterial growth is stimulated by fermentation. The major fermentation products of carbohydrates are the short-chain fatty acids (SCFA) (primarily acetate, propionate and butyrate), $CO_2$, $H_2$ and $CH_4$. Fermentation of prebiotics to SCFAs is central to many of the proposed mechanisms for health effects provided by prebiotics. Prebiotics have generally been observed to increase lactate and acetate concentrations, suggesting fermentation by lactic acid bacteria and bifidobacteria (8). However, propionate and butyrate are also produced during prebiotic fermentation, indicating that other members of the microflora also utilise these substrates. In investigations directly comparing SCFA production from various prebiotics, differences in the SCFA-profiles have been observed (64). Thus, different prebiotics are metabolised by the colonic microflora to different combinations of end products with possibly varying effects on human health.

### Effect of oligosaccharides on bacterial composition

A long-held belief, originating probably with Metchinikoff at the turn of the century, is that some gut bacteria are beneficial to health, whilst others may be harmful. Potentially health promoting bacteria are thought to include principally the bifidobacteria and lactobacilli.

Different approaches are currently used to obtain a bacterial composition with high numbers of these beneficial bacteria. Probiotics have been variously defined, but for purposes of human nutrition probiotics can be defined as 'a living microbial food ingredient that is beneficial to health. By definition, prebiotics are non-digestible food ingredients which beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve health (4). Another approach is the use of synbiotics. A synbiotic has been defined as a mixture of probiotics and prebiotics that beneficially affect the host by improving the survival of implantations of live microbial dietary supplements in the gastrointestinal tract by selectively stimulating the growth and/or activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare (4).

In attempts to assess the ability of oligosaccharides to selectively stimulate the proliferation of beneficial organisms in the colon, a range of *in vitro* models are being employed. Various commercially available oligosaccharides have been fermented by pure cultures in simple static/batch cultures. Most of the data have been obtained using differing procedures which may produce differing results. To determine the ability of a certain strain to ferment a carbohydrate, two methods are generally used. In the first method, only the pH shift in the medium is measured and these values are compared to a carbohydrate-free medium and a medium with glucose as a negative and positive control, respectively. Growth measurements are very useful. However, one must be sure that the oligosaccharide-mixtures used do not contain contaminating digestible material. Final proof for oligosaccharide fermentation can be obtained by following the fermentative degradation of the oligosaccharides with HPLC-techniques. Because the colon is a complex ecosystem where several interactions between the bacteria might occur, the bifidogenic properties of oligosaccharides have not only been studied in pure cultures but have been evaluated in more sophisticated, multistage intestinal models (66). However, ultimate proof of selective stimulation of beneficial bacteria in humans is required. Bifidogenic effect of lactulose (5); fructooligosaccharides (6, 7); galactooligosaccharides (8, 9); raffinose(10); lactosucrose (11, 12); isomaltooligosaccharides (13) and xylooligosaccharides (66) have been reported in healthy volunteers or patients.

## Effect of oligosaccharides on bacterial metabolism

In addition to the selective stimulation of growth of beneficial bacteria in the colon by prebiotics, these may also stimulate carbohydrate metabolism. The utilisation of oligosaccharides must be mediated by the hydrolysing enzymes they produce. For the metabolism of oligosaccharides, the bacterial species have to produce glycosidases in order to degrade the oligosaccharides into mono- or disaccharides which can be transported into the cell where they can further be metabolised towards SCFA. Rarely bacterial uptake systems are being reported for sugars with a degree of polymerisation > 2 (67). Many strains of

bifidobacteria produce glycolytic enzymes which hydrolyse a wide variety of substrates. In addition, other intestinal bacteria possess glycolytic enzymes (see table 5).

**Table 5.** Characterised glycosidases and glycanases of colonic bacteria.

| Dietary carbohydrates | Enzymes | Bacterial species |
|---|---|---|
| NeuAc($\alpha$2-3)Gal($\beta$1-4)Glc | Neuramidase | Bifidobacteria |
| ß-Glucooligosaccharides | ß-glucosidase | Bifidobacteria (68), Bacteroides (69, 70) |
| $\alpha$-Glucooligosaccharides | $\alpha$-glucosidase | Bifidobacteria (71), Bacteroides (70) |
| Fructooligosaccharides | ß-fructofuranosidase/ fructanase | Bifidobacteria (72, 73, 74) Clostridia (75) Bacteroides (76) |
| $\alpha$-Galactooligosaccharides | $\alpha$-galactosidase | Bifidobacteria (77, 78, 79) Lactobacilli (80) Bacteroides (81) |
| ß-Galactooligosaccharides | ß-galactosidase | Bifidobacteria (82) |
| (Arabino)xylan | Endo-xylanase, $\alpha$-arabinofuranosidase, ß-xylosidase | Bacteroides (83) |
| Arabinogalactan type II | Arabinogalactanase, $\alpha$-arabinofuranosidase, ß-galactosidase | Bifidobacteria (82) |

The conditions in the large intestine and the regulation mechanisms of the bacteria will determine which bacteria will be able to utilise a specific substrate.

## AIM AND OUTLINE OF THE THESIS

The research described in this thesis was part of a multi-disciplinary research program at the Wageningen Agricultural University on the role of non-digestible oligosaccharides in human and animal nutrition. Four research groups were involved in the project: Human Nutrition and Epidemiology, Animal Nutrition, Food Microbiology and Food Chemistry. The main objective of the research done at the Food Chemistry group was to establish the relation between the chemical structure of non-digestible oligosaccharides and their fermentability by intestinal bacteria. Therefore, several structurally different series of oligosaccharides needed to be produced and fractionated and their structure elucidated in order to obtain well-defined oligosaccharides. Secondly, the fermentation of the oligosaccharides by different intestinal bacteria had to be evaluated in order to obtain information on their capability to degrade and/or ferment oligosaccharides. Thirdly, some of the oligosaccharide degrading enzymes of

bacteria which are capable of fermenting oligosaccharides had to be purified and characterised to obtain more information on the activity, specificity and regulation of these enzymes. The acquired knowledge can then be used to design procedures for the tailor-made production of oligosaccharides.

Chapter 2 describes the production of structurally different oligosaccharides from plant polysaccharides and their fermentation by various intestinal bacteria in comparison to the fermentation of the parent polysaccharide.

In chapter 3 and 4, two different arabinofuranohydrolases from *Bi. adolescentis* -- which enable this species to be one of the few bacteria to utilise arabinoxylooligosaccharides completely -- were studied in more detail and were found to be new types of enzymes.

The fermentation of stachyose, an α-galactooligosaccharide utilised by *Bi. adolescentis*, and purification of the α-galactosidase involved in the degradation of this substrate is described in chapter 5. This purified α-galactosidase was used for the production of new types of α-galactosides that may function as prebiotics. Chapter 6 gives the results of our studies on the effect of transgalactooligosaccharides on the growth and enzyme-regulation of *Bi. adolescentis* and a study of the enzymes involved in the degradation of these transgalactooligsoaccharides.

In chapter 7, the effect of transgalactooligsoaccharides and a hydrolysate of soy arabinogalactans -containing both oligomeric and polymeric material- on the growth of bacteria in faecal slurry and their effect on the formation of oligosaccharide degrading enzymes was studied.

Chapter 8 reports on the effect of transgalactooligosaccharides and fructooligosaccharides on glycosidase levels in faeces of healthy volunteers and investigates whether glycosidase levels are markers for prebiotic effects.

Finally, in chapter 9, the main findings are summarised and discussed, and perspectives for the future are given.


## REFERENCES

1    Fuller R (1989) Probiotics in man and animals. J Appl Bacteriol 66: 365-378

2    Fooks LJ, Fuller R, Gibson GR (1999) Prebiotics, probiotics and human gut microbiology. Int Dairy J 9: 53-61

3    Crittenden RG, Tannock GW (1999) Prebiotics. In: Tannock GW Probiotics - A critical review – Introduction: 141-156 Horizon scientific press, Wymondham, UK.

4    Gibson GR, Roberfroid MB (1995) Dietary modulation of the human colonic microbiota. J Nutr 125: 1401-1412

5    Ballongue J, Schumann C, Quignon P, Vonk RJ (1997) Effects of lactulose and lactitol on colonic microflora and enzymatic activity. Scand J Gastroenterol 32 Supplement 222: 41-44

6   Hidaka H, Eida T, Takizawa T, Tokunaga T, Tashiro Y (1986) Effects of fructooligosaccharides on intestinal flora and human health. Bifid Microf 5: 37-50

7   Bouhnik Y, Vahedi K, Achour L, Attar A, Salfati J, Pochart P, Marteau P, Flourie B, Bornet F, Rambaud JC (1999) Short-chain fructooligosaccharide administration dose-dependently increases fecal bifidobacteria in healthy humans. J Nutr 129: 113-116

8   Bouhnik Y, Flourie B, D'Agay-Abensour L, Pochart P, Gramet G, Durand M, Rambaud JC (1997) Administration of transgalacto-oligosaccharides increases fecal bifidobacteria and modifies colonic fermentation metabolism in healthy humans. J Nutr 127: 444-448

9   Ito M, Deguchi Y, Matsumoto K, Kimura M, Onodera N, Yajima T (1993) Influence of galactooligosaccharides on the human fecal microflora. J Nutr Sci Vitamin 39: 635-640

10  Benno Y, Endo K, Shiragami N, Sayama K, Mitsuoka T (1987) Effects of raffinose intake on human fecal microflora. Bifid Microf 6: 59-63

11  Yoneyama M, Mandai T, Aga H, Fujii K, Sakai S, Katayama Y (1992) Effects of 4-G-beta-D-galactosylsucrose (lactosucrose) intake on intestinal flora in healthy humans. J Jpn Soc Nutr Food Sci 45: 101-107

12  Ohkusa T, Ozaki Y, Sato C, Mikuni K, Ikeda H (1995) Long-term ingestion of lactosucrose increases *Bifidobacterium sp* in human fecal flora. Digestion 56: 415-420

13  Kaneko T, Kohmoto T, Kikuchi H, Shiota M, Iino H, Mitsuoka T (1994) Effects of isomaltooligosaccharides with different degrees of polymerization on human fecal bifidobacteria. Biosc Biotechnol Biochem 58: 2288-2290

14  Okazaki M, Fujikawa S, Matsumoto N (1990) Effect of xylooligosaccharide on the growth of bifidobacteria. Bifid Microf 9: 77-86

15  Cummings JH, Roberfroid MB, Andersson H, Barth C, Ferro Luzzi A, Ghoos Y, Gibney M, Hermonsen K, James WPT, Korver O, Lairon D, Pascal G, Voragen AGJ (1997) A new look at dietary carbohydrate: chemistry, physiology and health. Eur J Clin Nutr 51: 417-423

16  Campbell JM, Bauer LL, Fahey GC Jr, Hogarth AJCL, Wolf BW, Hunter DE (1997) Selected fructo-oligosaccharide (1-kestose, nystose, and 1F-beta-fructofuranosylnystose) composition of foods and feeds. J Agric Food Chem 45: 3076-3082

17  Kuo TM, Van Middlesworth JF, Wolf WJ, Middlesworth JF van (1988) Content of raffinose oligosaccharides and sucrose in various plant seeds. J Agric Food Chem 36: 32-36

18  Saini HS, Gledstoe JS (1986) Variability in the total and component galactosyl sucrose oligosaccharides of *Lupinus* species. Aust J Agric Res 37: 157-166

19  Weston RJ, Brocklebank LK (1999) The oligosaccharide composition of some New Zealand honeys. Food Chem 64: 33-37

20  Thurl S, Muller-Werner B, Sawatzki G (1996) Quantification of individual oligosaccharide compounds from human milk using high-pH anion-exchange chromatography. Anal Biochem 235: 202-206

21  Lehtonen P, Hurme R (1994) Liquid chromatographic determination of sugars in beer by evaporative light scattering detection. J Inst Brew 100: 343-346

22  Castino M (1972) Residual sugars in wines. Vini Ital 14: 69-73 & 76-80

23  Rodionova NA, Kilimnik AYu, Kaprel'yants LV, Serednitskii PV, Martinovich LI, Zagustina NA, Bezborodov AM (1995) Enzyme preparations from mycelial fungi that cleave cereal grain polymers. Appl Biochem Microbiol 31: 370-376

24  Playne MJ, Crittenden R (1996) Commercially available oligosaccharides. BIDFD 313: 10-22

25  Prieto PA, Fletcher SD, Cummings RD, Kopchick JJ, Mukerji P, Moremen KW and Pierce JM (1999) Transgenic non-human mammals producing oligosaccharides and glycoconjugates. United States Patent: 5892070

26  Ly HD, Wither SG (1999) Mutagenesis of glycosidases. Annu Rev Biochem 68: 487-522

27  McCarter JD, Withers SG (1994) Mechanisms of enzymatic glycoside hydrolysis. Curr Opin Struct Biol 4: 885-892

28  Hidaka H, Hirayama M, Sumi N (1988) A fructooligosaccharide-producing enzyme from *Aspergillus niger* ATCC 20611. Agric Biol Chem 52: 1181-1187

29  Hirayama M, Nishizawa K, Hidaka H (1993) Production and characteristics of fructooligosaccharides. In: Fuchs (ed) Inulin and Inulin-containing crops. Elsevier, Amsterdam, The Netherlands

30  Zarate S, Lopez-Leiva MH (1990) Oligosaccharide formation during enzymatic lactose hydrolysis: a literature review. J Food Protection 53: 262-268

31  Onishi N, Yamashiro A, Yokozeki K (1995) Production of galacto-oligosaccharide from lactose by *Sterigmatomyces elviae* CBS8119. Appl Environ Microbiol 61: 4022-4025

32  Asp NG, Burval A, Dahlquist A, Hallgren P, Lundblad A (1980) Oligosaccharide formation during hydrolysis of lactose with *Saccharomyces lactis* lactase (MaxilactRegistered) II Oligosaccharide structures. Food Chem 5: 147-153

33  Toba T, Yokota A, Adachi S (1985) Oligosaccharide structures formed during the hydrolysis of lactose by *Aspergillus oryzae* beta-galactosidase. Food Chem 16:147-162

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

35  Usui T, Morimoto S, Hayakawa Y, Kawaguchi M, Murata T, Matahira Y, Nishida Y (1996) Regioselectivity of beta-D-galactosyl-disaccharide formation using the beta-D-galactosidase from *Bacillus circulans*. Carbohydr Res 285: 29-39

36  Yamashita K, Kobata A (1974) Oligosaccharides of human milk V Isolation and characterisation of a new trisaccharide, 6'-galactosyllactose. Arch Biochem Biophys 161:164-170

37  Mahony RR (1998) Galactosyl-oligosaccharide formation during lactose hydrolysis: a review. Food Chem 63: 147-154

38  Paul F, Lopez-Munguia A, Remoud M, Pelenc V, Monsan P (1988) Procédé de préparation enzymatique d'oligodextranes utiles dans la fabrication de substituts du sucre et nouveaux oligodextranes. French patent: 2626583

39    Dong Hyun Kim, Yong Jin Choi, Seung Koo Song, Jong Won Yun (1997) Production of inulo-oligosaccharides using endo-inulinase from a *Pseudomonas sp*. Biotechnol Lett 19: 369-371

40    Vogel M, Fuchs A (1993) A process for the production of inulin and its hydrolysis products from plant material. Inulin and inulin-containing crops Proceedings of the international congress on food and non-food applications of inulin and inulin-containing crops. Wageningen, Netherlands, 17-21 February (1991): 65-75

41    Park JP, Kim DH, Kim DS, Yun JW (1998) Enzymatic production of inulo-oligosaccharides from chicory juice. Biotech Lett 20: 385-388

42    Coussement P (1995) [Raftilose and Raftiline: a new generation of dietary fibre] Raftilose und Raftiline: eine neue Generation von Ballaststoffen. Deutsche Milchwirtschaft 46: 1060-1062

43    Chen CS, Chen JL, Lin TY (1997) Purification and characterisation of a xylanase from *Trichoderma longibrachiatum* for xylo-oligosaccharide production. Enzyme Microbiol Technol 21: 91-96

44    Tokuda H, Sato K, Nakanishi K (1998) Xylo-oligosaccharide production by *Aspergillus oryzae* 13 immobilized on a nonwoven fabric. Biosc Biotechnol Biochem 62: 801-803

45    Kofod LV, Mathiasen TE, Heldt-Hansen HP, Dalbøge H (1995) Application of cloned monocomponent carbohydrases for modification of plant materials. In: Petersen SB, Svensson B, Pedersen S (eds) Carbohydrate Bioengineering. Progress Biotechnol 10: 321-342, Elsevier Amsterdam, The Netherlands

46    Albertsheim P, Darvill AG, O'Neill MA, Schols HA, Voragen AGJ (1996) An Hypothesis: The same six polysaccharides are components of the primary cell walls of al higher plants. In: Visser J, Voragen AGJ (eds), Pectin and pectinases. Progress Biotechnol 14: 47-56, Elsevier, Amsterdam, The Netherlands

47    Yasuda E, Takaku H, Matsumotot H (1986) Japanese Patent, 61-212296

48    Hayashi S, Hinotani T, Takasaki Y, Imada K (1994) The enzymatic reaction for the production of panose and isomaltose by glucosyltransferase from *Aureobasidium*. Lett Appl Microbiol 19: 247-248

49    Trowell HC, Southgate DAT, Wolever TMS, Leeds AR, Gassull MA, Jenkins DJA (1976) Dietary fibre redefined. Lancet i: 1, 967

50    Asp NG (1990) In: Southgate DAT, Waldron K, Johnson IT, Fenwick GR (eds) Dietary fibre: chemical and biological aspects. Roy Soc Chem Special Publication 83: 327-336 Hardback, Cambridge, UK

51    Prosky L, Lee SC (1997) Classification of complex carbohydrates. Adv Exp Med Biol 427: 55-61

52    Quemener B, Thibault JF, Coussement P (1994) Determination of inulin and oligofructose in food products, and integration in the AOAC method for measurement of total dietary fibre. Lebensm-Wiss Technol 27: 125-132

53    Deutch MJ (1997) Vitamins and other nutrients In: Cunniff P (ed) Official methods of analysis of AOAC international. Vol 2: 70-71 Gaithersburg: AOAC International

54  Churms SC (1990) Recent developments in the chromatographic analysis of carbohydrates. J Chromatogr 500: 555-583

55  Corradini C, Canali G, Nicoletti I (1997) Application of HPAEC-PAD to carbohydrate analysis in food products and fruit juices. Semin Food Anal 2: 99-111

56  Schols HA, Voragen AGJ, Bruggink C (1992) Analysis of carbohydrates by High-Performance Anion-Exchange Chromatography-pulsed amperometric detection: an additional possibility or a breakthrough? VMT 25: 30-33

57  Schols HA, Van Laere KMJ, Voragen AGJ (1995) Determination of oligosaccharides In: Proceedings in recent progress in the analysis of dietary fibre. European Commission, Luxembourg: 53-59

58  Lee YC (1990) High-performance anion-exchange chromatography for carbohydrate analysis. Anal Biochem 189: 151-162

59  Thomas J, Mort AJ (1994) Continuous postcolumn detection of underivatized polysaccharides in High-performance Liquid Chromatography by reaction with permanganate Anal Biochem 223: 99-104

60  Niessen WMA, van der Hoeven RAM, van der Greef J, Schols HA, Voragen AGJ, Bruggink C (1993) Recent progress in high performance anion-exchange chromatography-thermospray mass spectrometry of oligosaccharides. J Chromatogr 647: 319-327

61  Mutter M, Renard CMGC, Beldman G, Schols HA, Voragen AGJ (1996) Characterisation of RG-degradation products of new RG-ases using RG-rhamnohydrolases and RG-galacturonohydrolase. In: Visser J, Voragen AGJ (eds), Pectin and pectinases, Progress in Biotechnology 14: 263-274 Elsevier, Amsterdam, The Netherlands

62  Tannock GW (1995) Normal microflora. An introduction to microbes inhabiting the human body. Chapman and Hall, London, UK

63  Cummings JH, Englyst HN (1987) Fermentation in the large intestine and the available substrates. Am J Clin Nutr 45: 1243-1255

64  Djouzi Z, Andrieux C (1997) Compared effects of three oligosaccharides on metabolism of intestinal microflora in rats inoculated with a human faecal flora. Br J Nutr 78: 313-324

64  Hartemink R (1999) Prebiotic effects of non-digestible oligosaccharides and polysaccharides. PhD Thesis, Wageningen University, Wageningen, The Netherlands

65  Okazaki M, Fujikawa S, Matsumoto N (1990) Effect of xylooligosaccharide on the growth of bifidobacteria. Bifid Microf 9: 77-86

66  Russell RR, Aduse-Opoku J, Sutcliffe IC, Tao L, Ferretti JJ (1992) A binding protein-dependent transport system in *Streptococcus mutans* responsible for multiple sugar metabolism. J Biol Chem 5: 4631-4637

68  Sakai K, Tachiki T, Kumagai H, Tochikura T (1986) Isolation and characterisation of two beta-D-glucosidases from *Bifidobacterium breve* 203. Agric Biol Chem 50: 2287-2293

69  Salyers AA, Palmer JK, Wilkins TD (1977) Laminarinase (beta-glucanase) activity in *Bacteroides* from the human colon. Appl Environ Microbiol 33: 1118-24

70  Berg JO, Lindqvist L, Nord CE (1980) Purification of glycoside hydrolases from *Bacteroides fragilis*. Appl Environ Microbiol 40: 40-47

71  Degnan BA, Macfarlane GT (1994) Synthesis and activity of alpha-glucosidase produced by *Bifidobacterium pseudolongum*. Curr Microbiol 29: 43-47

72  Muramatsu K, Onodera S, Kikuchi M, Shiomi N (1994) Substrate specificity and subsite affinities of beta-fructofuranosidase from *Bifidobacterium adolescentis* G1. Biosc Biotechnol Biochem 58: 1642-1645

73  Imamura L, Hisamitsu K, Kobashi K (1994) Purification and characterisation of beta-fructofuranosidase from *Bifidobacterium infantis*. Biol Pharm Bull 17: 596-602

74  Muramatsu K, Onodera S, Kikuchi M, Shiomi N (1992) The production of beta-fructofuranosidase from *Bifidobacterium spp*. Biosc Biotechnol Biochem 56: 1451-1454

75  Ishimoto M, Nakamura A (1997) Purification and properties of beta-fructofuranosidase from *Clostridium perfringens*. Biosc Biotech Biochem 61: 599-603

76  Blatch GL, Woods DR (1993) Molecular characterisation of a fructanase produced by *Bacteroides fragilis* BF 1. J Bacteriol 175: 3058-3066

77  Roy D, Blanchette L, Savoie L, Ward P, Chevalier P (1992) alpha- and beta-Galactosidase properties of *Bifidobacterium infantis*. Milchwiss 47: 18-21

78  Roy D, Chevalier P, Ward P, Savoie L (1991) Sugars fermented by *Bifidobacterium infantis* ATCC 27920 in relation to growth and alpha-galactosidase activity. Appl Microbiol Biotechnol 34: 653-655

79  Sakai K, Tachiki T, Kumagai H, Tochikura T (1987) Hydrolysis of $\alpha$-D-galactosyl oligosaccharides in soy by $\alpha$-D-galactosidase of *Bifidobacterium breve* 203. Agric Biol Chem 51: 315

80  Mital BK, Shallenberger RS, Steinkraus KH (1973) alpha -Galactosidase activity of Lactobacilli. Appl Microbiol 26: 783-788

81  Gherardini F, Babcock M, Salyers AA (1985) Purification and characterisation of two alpha-galactosidases associated with catabolism of guar gum and other alpha-galactosides by *Bacteroides ovatus*. J Bacteriol 161: 500-506

82  Tochikura T, Sakai K, Fujiyoshi T, Tachiki T and Kumagai H (1986) *p*-nitrophenyl glycoside-hydrolysing activities in bifidobacteria and characterisation of ß-galactosidase of *Bifidobacterium longum* 401. Agric Biol Chem 50: 2279-2286

83  Whitehead TR (1995) Nucleotide sequences of xylan-inducible xylanase and xylosidase/arabinosidase genes from *Bacteroides ovatus* V975. Biochim Biophys Acta 1244: 239-241

# 2

# Fermentation of plant cell wall derived polysaccharides and their corresponding oligosaccharides by intestinal bacteria

New types of non-digestible oligosaccharides were produced from plant cell wall polysaccharides, and the fermentation of these oligosaccharides and their parental polysaccharides by relevant individual intestinal species of bacteria was studied. Oligosaccharides were produced from soy arabinogalactan, sugar beet arabinan, wheat flour arabinoxylan, polygalacturonan, and a rhamnogalacturonan fraction from apple. All of the tested substrates were fermented to some extent by one or more of the individual species of bacteria tested. The tested *Bacteroides* spp. were able to utilise plant cell wall derived oligosaccharides besides their reported activity toward plant polysaccharides. The tested *Bifidobacterium* spp. were also able to utilise the rather complex plant cell wall derived oligosaccharides in addition to the bifidogenic fructooligosaccharides. Strains belonging to *Clostridium* spp., *Klebsiella* spp., and *Escherichia coli* fermented some of the selected substrates *in vitro*. These studies do not allow prediction of the fermentation *in vivo* but give valuable information on the fermentative capability of the tested intestinal strains.

## INTRODUCTION

Non-digestible oligosaccharides (NDOs) are oligosaccharides, which escape digestion in the upper gastrointestinal tract. According to the IUB-IUPAC nomenclature, oligosaccharides are defined as saccharides containing between 3 and 10 sugar moieties (Voragen, 1998). The oligosaccharides, fructooligosaccharides, and transgalactooligosaccharides belong to the group of prebiotics meaning that they are non-digestible food ingredients, that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improve health (Gibson and Roberfroid, 1995). Mixtures of oligosaccharides which combine the effect of stimulating the body's own bacteria and those added (probiotics) are considered as synbiotics (Knorr, 1998).

Some NDOs occur naturally in various plants (Campbell et al, 1997), processed foods and in human breast milk (Thurl et al, 1996). Non-digestible oligosaccharides, as well as non-starch-polysaccharides (NSP), resistant starch and mucin can all be regarded as substrate for the intestinal species of bacteria (Vercellotti et al, 1977). NDOs are commercially produced by extraction, chemical condensation (Dendene et al, 1994), transglycosylation reactions (Smart, 1993; Barthomeuf and Pourrat, 1995) or controlled hydrolysis of polysaccharides (Coussement, 1995; Yun et al, 1997). The various types of dietary carbohydrates might influence the growth of specific bacteria species, their metabolic activity, short chain fatty acid (SCFA), production and rate of fermentation in different ways dependent on their structures.

Species often involved in the breakdown of carbohydrates belong to the genera *Bacteroides*, *Bifidobacterium*, *Ruminococcus*, *Eubacterium*, *Lactobacillus,* and *Clostridium.* Some species (mainly *Bacteroides*) are regarded as utilising mainly NSP while others grow by crossfeeding on smaller fragments (often oligosaccharides) produced by primary NSP degraders (Macfarlane and Cummings, 1991). Bifidobacteria have been reported to be mainly oligosaccharide utilisers, although some of them are also able to ferment polysaccharides (Crociani et al, 1994).

Products formed during fermentation in the colon from NSP have been the subject of many studies, whereas almost no data are available on the fermentation of oligosaccharides derived from NSP. Most of the research has focused on the influence of fructooligosaccharides and galactooligosaccharides on the intestinal flora (Ito et al, 1993; Alles et al, 1996; Buddington et al, 1996; Garleb et al, 1996). However plant cell wall polysaccharides are also an interesting potential source for the production of oligosaccharides covering a wide range of different structures. Plant cell wall polysaccharides are present in large amounts as fibre-rich by-products, e.g., cereal bran, fruit pomace, sugar beet pulp, potato fibre, and press cake of oleaginous seeds. If required specific extractions can results in polysaccharides which can be hydrolysed towards oligosaccharides. Little attention is paid to the possible use of plant cell wall derived oligosaccharides as prebiotic substrate. As these types of oligosaccharides can be formed during production and/or processing of food (e.g., formation of

arabinoxylooligosaccharides during bread making in case endo-xylanases are added as bread-improvers, in fruit juices, purees and nectars if enzymes are used, isomaltooligosaccharides in beer) they might be an important prebiotic substrate. Also adding these types of oligosaccharides to the diet might have interesting effects on the composition and/ or activity of the bacterial flora. Fermentation of complex carbohydrates is assumed to be a result of a combined action of several bacteria: however, knowledge on how individual intestinal species of bacteria utilise complex poly- and oligosaccharides is important to better understand their fermentation in the colon.

The aim of this study was to produce various structurally different oligosaccharides from plant cell wall polysaccharides and to study the influence of the chemical structure of these oligosaccharides on the fermentation behaviour by various intestinal bacteria. Further, this behaviour was compared to the fermentation behaviour of parental polysaccharides and commercially available oligosaccharides. To obtain more information on the fermentation capabilities of various intestinal strains, this study was performed with pure cultures and well-defined substrates.

## MATERIALS AND METHODS

### Materials

Wheat flour arabinoxylan was obtained from Megazyme (International Ireland Ltd, Wicklow, Ireland). Sorghum arabinoxylan was obtained as described by Verbruggen et al, (1995). A rhamnogalacturonan-enriched fraction was isolated from apple liquefaction juice and was subsequently saponified according to the method of Schols et al (1990). Soy arabinogalactan was obtained on a small scale as described by Huisman et al (1998), and a large amount of soy arabinogalactan was a gift from Novo Nordisk Ferment AG (Dittingen, Switzerland). Sugar beet arabinan was a gift from British Sugar (Peterborough, UK). Polygalacturonic acid was obtained from ICN (Costa Mesa, CA). Xylooligosaccharides were a gift from Suntory Ltd. (Japan). Fructooligosaccharides obtained from inulin by controlled hydrolysis (Raftilose P95) were kindly provided by ORAFTI (Tienen, Belgium).

### Enzymes

Polygalacturonase was purified from *Kluyveromyces fragilis* (Versteeg, 1979). Rhamnogalacturonan hydrolase was purified from the commercial preparation Ultra Sp produced with *Aspergillus aculeatus* (Novo Nordisk A/S, Bagsvaerd, Denmark). An endo-xylanase was purified from *Aspergillus tubigensis* (Graaf et al, 1994). Endo-galactanase and endo-arabinanase were cloned from *A. aculeatus* and were kindly provided by Novo Nordisk A/S (Bagsvaerd, Denmark). The cloned enzymes may not be completely pure since the host

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micro-organism produces low amounts of its own enzymes in addition to the genetically introduced enzyme.

## Preparation of oligosaccharides from plant cell wall polysaccharides

A solution of polymer (1% w/v) was incubated with an appropriate amount of suitable endo-glycanase and the degradation was followed by High-Performance Anion-Exchange Chromatography (HPAEC) and High-Performance Size-Exclusion Chromatography (HPSEC) (see analytical methods). After inactivation of the enzyme (10 min, 100 °C), the solution was concentrated under reduced pressure. Mostly, the concentrated digest required centrifugation (14000 × g, 5 min) to remove insoluble material. The supernatants were fractionated on a Sephadex G10 column (600 × 50mm), (Amersham Pharmacia Biotech, Uppsala, Sweden) or two columns (600 × 26 mm) packed in series with Fractogel TSK HW-40 (S) (25-40 µm, Merck, Darmstadt, Germany) thermostated at 60 °C, or a BioGel P-2 column (1000 × 26 mm) (200-400 mesh, Bio-Rad Laboratories, Hercules, CA) thermostated at 60 °C. Samples up to 5 mL were applied on the column and eluted with distilled water (5 mL/min, 2.5 mL/min, 0.5 mL/min respectively for G10, TSK and BioGel material) using a Pharmacia Whiled system equipped with a Pharmacia P50 pump. A Shodex RI-72 detector was used to monitor the refractive index. The fractions were analysed for oligosaccharides by HPAEC or HPSEC and appropriate fractions were pooled.

## Bacterial strains

Eighteen bacterial strains were selected from the culture collection in our department. Most strains were of human origin; some strains originated from porcine feces (Hartemink et al, 1996).

## Oligosaccharide and polysaccharide fermentation

The selected strains were screened for their ability to ferment structurally different oligosaccharide and polysaccharide fractions. Strains were pre-grown in thioglycollate broth (Oxoid, Unipath LTD, Basingstoke, Hampshire, UK). The sugar free thioglycollate medium, as well as the oligosaccharide or polysaccharide solutions were sterilized separately for 15 min at 121 °C. Thioglycollate supplemented with 0.5% oligosaccharides or polysaccharides (w/v) (1 mL) was inoculated with 10% (w/v) of an overnight full grown strain for 48 h at 37°C in an anaerobic chamber with an atmosphere consisting of $CO_2$ (10%), $H_2$ (10%) and $N_2$ (10%). After anaerobic incubation, the pH was measured using a micro-pH meter (Sentron, Roden, The Netherlands). The experiments were performed in duplicate. The changes in content of residual polysaccharides and oligosaccharides, and formation of reaction products were measured by HPAEC and HPSEC. For HPAEC and HPSEC analyses, the cultures were centrifuged and the supernatant was diluted 10 times with $H_2O$ and boiled for 5 min to

inactivate enzymatic activity. The strains were checked microscopically for purity before and after fermentation.

## Analytical methods

The HPAEC system consisted of a Dionex Bio-LC GPM-II quaternary gradient module (Dionex Corporation, Sunnyvale, CA) equipped with a Dionex CarboPac PA-100 column (4 × 250 mm) in combination with a CarboPac PA-100 guard column (3 × 25 mm). Samples (20 µL) were injected using a Spectra Physics SP8880 autosampler. The oligomers were analysed using a gradient of sodium acetate in 100 mmol.L$^{-1}$ sodium hydroxide. For RGAOS, the gradient was as follows: 0 to 5 min, 0 mM sodium acetate; 5 to 35 min, 0 - 430 mmol.l$^{-1}$ sodium acetate; 35 to 40 min, 430 to 1000 mmol.L$^{-1}$ sodium acetate. For AOS, the elution involved a linear gradient of 0-500 mmol.L$^{-1}$ sodium acetate in 100 mmol.L$^{-1}$ NaOH for 40 min. For GAOS, the elution involved a linear gradient of 200-700 mmol.L$^{-1}$ sodium acetate in 100 mmol.L$^{-1}$ NaOH for 40 min. For AGOS the elution involved a linear gradient of 0-400 mmol.L$^{-1}$ sodium acetate in 100 mmol.L$^{-1}$ NaOH for 40 min. Elution of AXOS was done with a linear gradient of 0-100 mmol.L$^{-1}$ sodium acetate in 100mmol.L$^{-1}$ NaOH for 5 min, followed by a linear gradient of 100-400 mmol.L$^{-1}$ sodium acetate in 100 mmol.L$^{-1}$ NaOH for 40 min. The elution of XOS involved a linear gradient of 0- 250 mmol.L$^{-1}$ sodium acetate for 30 min. All gradients ended with 5 min 1000 mmol.L$^{-1}$ sodium acetate and a reequilibration with 100 mmol.L$^{-1}$ NaOH for 15 min. The eluant (1 mL/min) was monitored using a Dionex PED detector in the pulsed amperometric detection (PAD) mode.

HPSEC was performed on a SP8800 HPLC (Spectra Physics) equipped with three Bio-Gel TSK-columns (each 300 × 7.5 mm) in series (40XL, 30XL, and 20XL; Bio-Rad labs) in combination with a TSK XL guard column (40 × 6 mm) and elution at 30 °C with 0.4 M acetic acid/ sodium acetate (pH 3) at 0.8 mL/min. The eluate was monitored using a Shodex SE-61 refractive index detector. The system was calibrated with pectins with molecular weights in the range of 10.000-100.000 Da (as determined by viscosimetry).

The polymeric material was analysed for sugar composition. Uronic acid was determined by the colorimetric *m*-hydroxybiphenyl assay (Ahmed and Labavitch, 1977). Neutral sugars were determined by GLC after pretreatment (1 h, 30 °C) with aqueous 72% $H_2SO_4$, followed by hydrolysis with 1 M $H_2SO_4$ (3 h, 100 °C) and conversion of the products into alditol acetates (Englyst and Cummings, 1984). The alditol acetates were analysed on a glass column (3 m × 2 mm i.d.), packed with Chrom WAW 80-100 mesh coated with 3% OV275 in a Carbo Erba Fractovap 2300 GC.

## RESULTS

### Production of new NDOs

#### Production of (arabino)galactooligosaccharides (AGOS) from soy

Dehulled soy beans were defatted, deproteinated, destarched and treated with NaOH according to Huisman et al (1998) to obtain an arabinogalactan enriched extract. The sugar composition of the arabinogalactan enriched polysaccharide fraction $(AGPS^1)$ obtained is given in Table 1.

**Table 1.** Sugar composition (mol %) of various cell wall polysaccharides and extracts

|             | AGPS[1] | AGPS[2] | APS | RGAPS | AXWPS | AXSPS |
|-------------|---------|---------|-----|-------|-------|-------|
| Rhamnose    | 2       | 2       | 5   | 16    | 0     | 0     |
| Fucose      | 3       | 2       | 0   | 0     | 0     | 0     |
| Arabinose   | 27      | 38      | 60  | 20    | 30    | 46    |
| Xylose      | 7       | 2       | 0   | 11    | 69    | 40    |
| Mannose     | 0       | 0       | 0   | 0     | 0     | 0     |
| Galactose   | 39      | 52      | 15  | 18    | 0     | 2     |
| Glucose     | 2       | 0       | 5   | 2     | 1     | 2     |
| Uronic acid | 20      | 4       | 15  | 33    | 0     | 10    |

AGPS: arabinogalactan enriched polysaccharide fraction; APS: arabinan enriched polysaccharide fraction; RGPS: rhamnogalacturonan enriched polysaccharide fraction; AXWPS: arabinoxylan polysaccharide from wheat flour; AXSPS: glucuronoarabinoxylan polysaccharide from sorghum

This fraction was mainly composed of galactose, arabinose, and galacturonic acid residues, but also some other sugar residues were present. This arabinogalactan extract was treated with various endo-glycanases for 1 h and 24 h, and the degradation of the polymer and subsequent oligosaccharide formation was analysed with HPSEC and HPAEC. Treating the extract for 24 h with a purified endo-galactanase resulted in the production of oligosaccharides with a degree of polymerisation (DP) of 2-5. An incubation time of 1 h resulted in the formation of oligomers with a DP ranging from 2 to 9.

Arabinogalactan obtained from Novo Nordisk $(AGPS^2)$ consisting of 52% galactose and 38% arabinose (Table 1) was used for the large-scale production of AGOS. $AGPS^2$ was incubated with the cloned endo-galactanase. The oligosaccharides formed eluted similar on HPAEC as those formed after incubation of the soy-extract with the purified endo-galactanase. The reaction products were applied onto a Sephadex G-10 column to remove the monomers and the remaining polymeric fraction. The mixture was fractionated using the BioGel P-2 column

in fractions containing di-, tri-, tetra-, penta-, hexa-, hepta-, octa- and nonasaccharides representing 22, 26, 16, 12, 9, 9, 3 and 3 % respectively of the oligosaccharide mixture. HPAEC analysis revealed that the different DP-fractions consisted of several types of oligosaccharides. The oligosaccharides present at the highest concentration in the various fractions were identified as galactooligosaccharides that were ß-1→4 connected to each other. The proposed structure of the oligosaccharides present in the mixture is given in Table 2.

### Production of arabinooligosaccharides (AOS) from sugar beet

Arabinan can be hydrolysed toward oligosaccharides by action of endo-arabinanases (Beldman et al, 1993). Linear α-1,5-arabinan extracted from sugar beet consisting mainly of arabinose (Table 1) was incubated with an endo-arabinanase. AOS with a DP of 2-10 were formed. The digest was applied onto a BioGel P-2 column, and the sugars eluting in the dimeric, trimeric, tetrameric and pentameric fraction were pooled. The AOS were identified using standards present in the laboratory as α-1→5 linked arabinose oligomers (Table 2). Due to the presence of some polygalacturonidase activity in the endo-arabinanase preparation, also some galacturonic acid (5% w/w) was present in the pooled mixture.

### Production of galacturonooligosaccharides (GAOS)

Linear GAOS were obtained by incubating polygalacturonic acid with a purified polygalacturonase from *Kluyveromyces fragilis* according to Versteeg (1979). Using HPSEC the degradation of the polygalacturonic acid was monitored. When the polymer was completely degraded toward oligomeric material, the reaction was stopped. The digest was dialyzed against Millipore deionized water to remove most of the monogalacturonic acid and digalacturonic acid and some trigalacturonic acid. The mixture obtained contained linear galacturonooligosaccharides with a DP ranging from 2 to10 (Table 2).

### Production of rhamnogalacturonooligosaccharides (RGAOS) from apple

From apple a highly branched rhamnogalacturonan enriched polysaccharide fraction (RGPS) was obtained as described by Schols et al (1990). This fraction was degraded by rhamnogalac-turonan hydrolase. The digest was fractionated on a Sephadex G-50 column according to Schols et al (1994). Fractions containing sufficient amounts of oligosaccharides were pooled and the structures of the oligosaccharides present in these pools were determined by comparing their elution pattern with the elution pattern of oligosaccharides with known structure (Schols et al, 1994). In Table 2 the structures of the RGAOS present in the mixture are given.

**Table 2.** Structure of the oligosaccharides derived from plant cell wall polysaccharides: (Gal)ₙ: galactooligosaccharides, (Gal)ₙ (Ara)ₙ: branched (arabino)galactooligosaccharides, (Ara)ₙ: arabinooligosaccharides, (GalA)ₙ : galacturonooligosaccharides, RGOS, rhamnogalacturonooligosaccharides., (Xyl)ₙ: xylooligosaccharides, AXOS: arabinoxylooligosaccharides

| | |
|---|---|
| **AGOS** | ß-D-Gal$p$-(1→4)-[ß-D-Gal$p$-(1→4)]ₙ-D-Gal$p$ with n= 0-5 <br> unidentified branched arabinogalactan derived oligosaccharides |
| **AOS** | α-L-Ara$f$-(1→5)-[α-L-Ara$f$-(1→5)]ₙ-L-Ara$f$ with n=0-3 |
| **GAOS** | α-D-GalA$p$-(1→4)-[α-D-GalA$p$-(1→4)]ₙ-D-GalA$p$ with n=0-7 |

**RGOS**

RG₁

α-Rha-(1→4)-α-GalA-(1→2)-α-Rha-(1→4)-GalA

RG₂

α-Rha-(1→4)-α-GalA-(1→2)-α-Rha-(1→4)-GalA
  ↓       ↓
 ß-Gal-(1,4)     ß-Gal-(1,4)

RG₃

α-Rha-(1→4)-α-GalA-(1→2)-α-Rha-(1→4)-α-GalA-(1→2)-α-Rha-(1→4)-GalA
  ↓       ↓         ↓
 ß-Gal-(1,4)    ß-Gal-(1,4)ₙ      ß-Gal-(1,4)ₘ

with either n=1 and m=0, or n=0 and m=1.

| | |
|---|---|
| **(Xyl)ₙ** | ß-Xyl$p$-(1→4)-[ß-Xyl$p$-(1→4)]ₙ-ß-Xyl$p$ n=0-4 |

**AXOS**

5.5

ß-Xyl$p$-(1→4)-ß-Xyl$p$-(1→4)-ß-Xyl$p$-(1→4)-Xyl$p$
         \
      α-Ara$f$(1,2)

5.1

    ß-Xyl$p$-(1→4)-ß-Xyl$p$-(1→4)-Xyl$p$
      /\
      / α-Ara$f$(1,2)
    α-Ara$f$(1,3)

6.1

ß-Xyl$p$-(1→4)-ß-Xyl$p$-(1→4)-ß-Xyl$p$-(1→4)-Xyl$p$
      /\
      / α-Ara$f$(1,2)
    α-Ara$f$(1,3)

8.1

ß-Xyl$p$-(1→4)-ß-Xyl$p$-(1→4)-ß-Xyl$p$-(1→4)-ß-Xyl$p$-(1→4)-Xyl$p$
     /     /\
     /     / α-Ara$f$(1,2)
  α-Ara$f$(1,3) α-Ara$f$(1,3)

9.1

ß-Xyl$p$-(1→4)-ß-Xyl$p$-(1→4)-ß-Xyl$p$-(1→4)-ß-Xyl$p$-(1→4)-Xyl$p$
     /\    /\
    / α-Ara$f$(1,2) / α-Ara$f$(1,2)
  α-Ara$f$(1,3) α-Ara$f$(1,3)

10.1

ß-Xyl$p$-(1→4)-ß-Xyl$p$-(1→4)-ß-Xyl$p$-(1→4)-ß-Xyl$p$-(1→4)-ß-Xyl$p$-(1→4)-Xyl$p$
     /\       /\
    / α-Ara$f$(1,2)   / α-Ara$f$(1,2)
  α-Ara$f$(1,3)    α-Ara$f$(1,3)

**Production of arabinoxylooligosaccharides (AXOS) from wheat-flour**

Arabinoxylan-enriched fractions were obtained from wheat flour and degraded with an endo-xylanase from *A. tubigensis*. The resulting digest was subsequently fractionated on Fractogel TSK. The fractions were analysed by HPAEC, and the oligomers present were identified using standards present in the laboratory (Gruppen et al, 1992). The fraction containing mainly double-branched oligosaccharides (Table 2) was used for further fermentation experiments.

Linear xylooligosaccharides (XOS) were obtained after partial purification of commercially available xylooligosaccharides. The commercial mixture contained 22% monosaccharides, which were removed by size-exclusion on a BioGel P-2 column. The di-, tri, and tetrameric fractions were pooled and contained, besides the linear ß1-4 linked xylooligosaccharides, also some other oligosaccharides (most likely glucooligosaccharides).

## Behaviour in the presence of isolated strains

Various plant cell wall polysaccharides and oligosaccharides were fermented by selected bacterial strains isolated from human and porcine feces. After anaerobic fermentation for 48 h, the pH was measured (Table 3). The amount of residual soluble polysaccharides or oligosaccharides was measured with HPSEC and HPAEC. The degree of fermentation of the polymers could be estimated by HPSEC from the shift in molecular weight or from the lowered amount of polymer left in the supernatant after fermentation. The presence of monomeric and oligomeric material after fermentation of the polymer was determined with HPAEC. The fermentation of the oligosaccharides was judged by comparing the HPAEC elution patterns of the oligosaccharides before and after fermentation. The thioglycolate broth and the oligosaccharide mixtures were also analysed separately to determine the origin of the observed peaks. This allowed specific determination of the degree of fermentation of the substrate by the various intestinal bacteria. In Table 4 the degradation of the polymers or oligomers by the various strains is given.

*Arabinogalactan enriched polysaccharide fraction (AGPS$^l$) and (arabino)galactooligosaccharides (AGOS)*

Species belonging to the different bacterial groups tested were able to ferment *AGPS$^l$*. Using HPSEC it was shown that 12 species of bacteria degraded the *AGPS$^l$* to some extent (Table 4); however, the degree of fermentation differed. In Figure 1, some HPSEC elution patterns obtained for various fermentation liquids are given. *Bacteroides ovatus* (*B. ovatus*) (Fig 1E) fermented the extract almost completely within the incubation time as can be seen when the pattern is compared with the HPSEC elution pattern of the untreated polymer (Fig 1A). *E. coli* (Fig 1D) degraded the polymer only to some extent; the molecular weight shifted from approximately 65 kDa to 20 kDa. *Lactobacilus acidophilus* (Fig 1C) and *Bifidobacterium breve* (*Bi. breve*) (Fig 1B) changed the elution behaviour of the *AGPS$^l$* only slightly. After 48

h fermentation no formed monomeric or oligomeric material could be detected in any of the *AGPS[1]* fermentations.
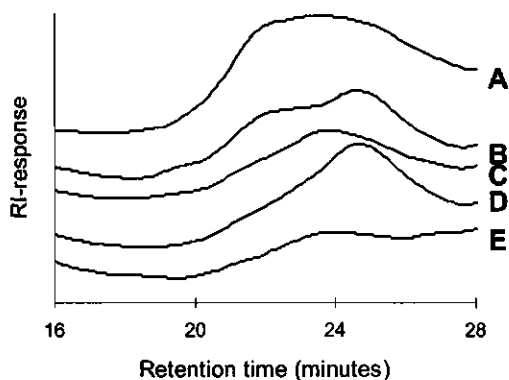


**Figure 1.** High-performance size-exclusion chromatography of arabinogalactan enriched polysaccharide fraction from soy before and after fermentation by various intestinal bacteria. A. Blank, B. *Bi. breve*, C. *L acidophilus*, D. *E. coli*, E. *B. ovatus*

A decrease in pH (see Table 3) of the medium was observed for almost all strains tested when grown on AGOS. In Figure 2 the HPAEC elution pattern of the AGOS before (Fig 2A) and after fermentation is given. Most of the tested strains showing a decrease in pH fermented the mixture completely (Fig 2C) as was monitored using HPAEC. *B. vulgatus* only slightly fermented AGOS Fig 2B). Some strains showed degradation of oligosaccharides but this degradation was not always accompanied with a drop in pH (Tables 3 and 4). It should be stated that the peaks still present in Figure 2C originate from the medium and not from the AGOS.
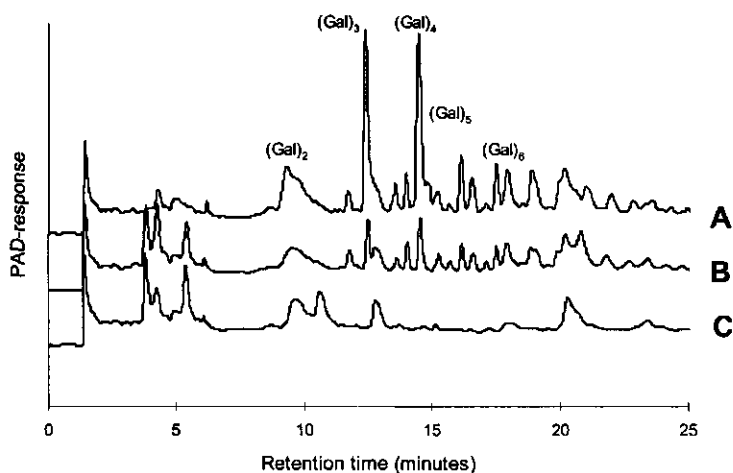


**Figure 2.** HPAEC analysis of (arabino)galactooligosaccharides before (A) and after fermentation *B. vulgatus* (B) and *L. acidophilus* (C)

## Arabinan enriched polysaccharide fraction (APS) and the arabinooligosaccharides (AOS)

The APS from sugar beet could degraded to some extend by *Bi. adolescentis*, *Clostridium beijerinckii* (*C. beijerinckii*), *C. clostridiiforme*, *C. ramosum*, *B. ovatus* and *B. thetoatoamicron* as measured by HPSEC (shift in $M_w$ from 32 to 22 kDa). The relative amount of polymer did not decrease for *C. beijerinckii* and *C ramosum* showing that these species only slightly hydrolysed the polymer but did not utilise it. *Bi. longum*, *C. clostridiiforme*, *B. vulgatus*, and *B. thetaiotaomicron* degraded this polymer and also decreased the amount of polymer, as measured by the change of area under the curve and a drop in pH. For the latter two species, arabinooligosaccharides still could be detected in the supernatant (Fig 3) showing that they could not utilise all of the oligosaccharides formed within the selected fermentation time. *Bi. longum* and *C. clostridiiforme* appeared to possess the enzyme system to fully utilise arabinan.
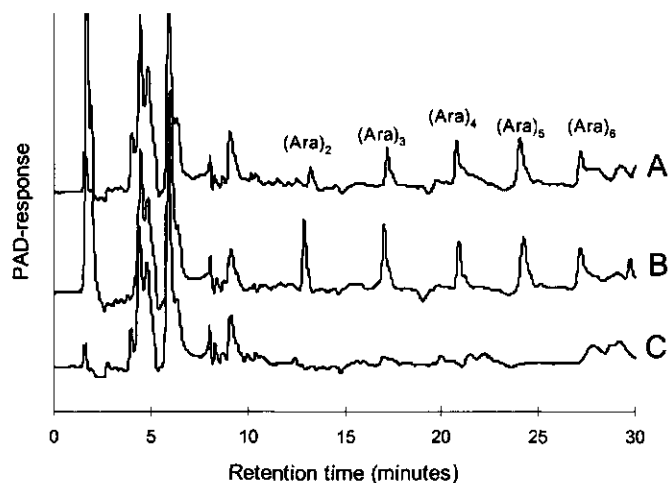


**Figure 3.** HPAEC elution pattern of arabinooligosaccharides formed in the supernatant before (C) and after fermentation of *B. thetaiotaomicron* (B) and *B. vulgatus* (A)

AOS of DP 2-6 were completely fermented by *Bi. longum* and *C. clostridiiforme*. *E. col*, *B. vulgatus*, *Bi. breve* and *Bi. adolescentis* fermented the smaller oligomer (DP 2-3). *C. beijerinckii* and *C. sartagoformum* slightly degraded the arabinotriose and arabinotetraose. The other species tested (Table 4) were unable to degrade the oligosaccharides.

***Rhamnogalacturonan enriched polysaccharide fraction (RGAPS) and (rhamno)galacturonooligosaccharides ((R)GAOS)***

RGAPS was fermented by a limited number of bacterial species only (Table 4). *B. ovatus* completely degraded the polymers while the other species tested were only able to degrade the population with the highest molecular weight.

RGAOS were completely fermented by the *Bacteroides* strains tested; *B. vulgatus, B. ovatus,* and *B. thetaiotaomicron.* In Figure 4 the elution profile is given of the RGAOS before and after incubation with *B. ovatus.* These species therefore probably produce galacturonidases, galactosidases and rhamnosidases when grown on these oligosaccharides. These species also lowered the pH of the supernatant after fermentation. The other species tested showed no activity toward RGAOS.
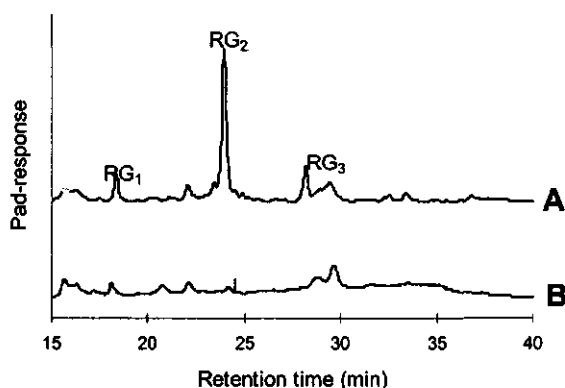


**Figure 4.** HPAEC elution pattern of rhamnogalacturonan-oligosaccharides before (A) and after (B) fermentation by *B. ovatus*

*C. clostridiiforme, C. ramosum,* and *B. ovatus* fermented linear GAOS. The other species tested did not show any degradation of the oligosaccharides. Since our substrates were only slightly buffered, the GAOS resulted in a pH-drop, and this complicates comparison with other substrates.

***Arabinoxylan polysaccharide from wheat flour (AXWPS), glucuronoarabinoxylan polysaccharide from sorghum (AXSPS) and (arabino)xylooligosaccharides (AXOS and XOS)***

Two arabinoxylans originating from different sources (wheat and sorghum) were selected for studying the fermentation of branched xylans. Wheat flour arabinoxylan consisted of arabinose and xylose while the polymer from sorghum (glucuronoarabinoxylan) contained glucuronic acids as substituents as well (Table 1); the polymer from sorghum was only partially water-soluble.

**Table 3.** Acidification of the medium after fermentation of various polysaccharides and oligosaccharides by several strains

| Bacteria | origin | Arabinogalactan | | arabinan | | (rhamno)galacturonan | | | (arabino)xylan | | | | FOS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AGPS | AGOS | APS | AOS | RGAPS | RGAOS | GAOS | AXWPS | AXSPS | AXOS | XOS | |
| | Blanc | 6.6 | 6.6 | 6.6 | 6.6 | 6.6 | 6.8 | 5.5 | 6.6 | 6.6 | 6.6 | 6.3 | 6.6 |
| *Bi. breve* | ATCC 15700 | 6.1 | 4.7 | 5.8 | 5 | 5.9 | 6.5 | nd | 6.3 | 5.7 | 5.9 | nd | 4.5 |
| *Bi. longum* | ATCC 15707 | 5.6 | 4.5 | 5.4 | 4.8 | 5.8 | 6.6 | 5.1 | 5.6 | 5.3 | 5.0 | 4.6 | 4.9 |
| *Bi. infantis* | ATCC 15697 | 6.0 | 4.4 | 6.0 | 6.1 | 6.0 | 6.6 | 5.2 | 6.1 | 6.0 | 5.9 | 5.1 | 4.5 |
| *Bi. adolescentis* | ATCC 15703 | 5.9 | 4.6 | 5.9 | 4.9 | 5.9 | 7.6 | 5.1 | 5.9 | 5.9 | 4.5 | 4.6 | 4.6 |
| *C. beijerinckii* | human feces | 6.8 | nd | 6.8 | 5.3 | 6.8 | 7.1 | 5.5 | 6.8 | 6.8 | 7.1 | 5.1 | 6.6 |
| *C. clostridiiforme* | human feces | 5.4 | 6.0 | 5.7 | 5.4 | 6.6 | 7.1 | 5.6 | 6.4 | 6.5 | 6.9 | nd | 5.5 |
| *C. ramosum* | human feces | 6.9 | 5.4 | 6.8 | 6.6 | 6.8 | 7.1 | 5.8 | 7.0 | 6.9 | 6.9 | 6.4 | 6.8 |
| *C. sporogenes* | human feces | 6.8 | >6 | 6.9 | 6.5 | 6.8 | 6.9 | 5.2 | 6.9 | 6.8 | 6.9 | 5.5 | 6.7 |
| *C. sartagoformum* | human feces | 6.6 | >6 | 7.2 | 5.7 | 6.8 | 6.8 | 5.4 | 6.8 | 6.8 | 6.9 | 5.1 | 6.6 |
| *C. perfringens* | human feces | 6.4 | >6 | 6.4 | 6.4 | 6.4 | 6.6 | 6.2 | 6.6 | 6.4 | 5.9 | 5.3 | 5.4 |
| *B. vulgatus* | ATCC 8482 | 6.1 | 5.2 | 5.7 | 6.1 | 5.9 | 4.9 | 5.1 | 6.4 | 6.1 | 5.2 | 5.6 | 5.1 |
| *B. ovatus* | ATCC 8483 | 5.6 | 5.2 | 5.7 | 6.2 | 5.6 | 5.7 | 5.1 | 5.6 | 5.6 | 5.0 | 5.3 | 5.4 |
| *B. thetaiotaomicron* | ATCC 29741 | 5.6 | 4.5 | 5.5 | 6.3 | 5.6 | 5.6 | 5.0 | 6.1 | 5.8 | 5.9 | 5.3 | 5.5 |
| *L. casei* | Yakult | 6.3 | 5.7 | 6.3 | 6.3 | 6.6 | 6.5 | 5.3 | 6.3 | 6.4 | 6.2 | 5.5 | 5.8 |
| *L. acidophilus* | swine feces | 6.5 | 5.5 | 6.6 | 6.3 | 6.6 | 6.5 | 5.3 | 6.7 | 6.5 | 6.7 | 4.9 | 6.4 |
| *L. fermentum* | swine feces | 6.7 | 5.2 | 6.2 | 6.2 | 6.7 | 5.9 | 5.1 | 6.7 | 6.7 | 6.3 | 4.5 | 6.6 |
| *E. coli* | human feces | 6.4 | 6.2 | 6.7 | 5.3 | 6.7 | 7.1 | 5.1 | 6.6 | 6.7 | 6.8 | 6.8 | 6.5 |
| *K. pneumoniae* | human feces | 6.6 | 4.8 | 6.8 | 7.1 | 6.8 | 6.9 | 5.6 | 6.7 | 6.8 | 6.0 | 6.0 | 5.8 |

AGPS: arabinogalactan enriched polysaccharide fraction; AGOS: (arabino)galactooligosaccharides; APS: arabinan enriched polysaccharide fraction; AOS: arabinooligosaccharides; RGPS: rhamnogalacturonan enriched polysaccharide fraction; RGAOS: rhamnogalacturonooligosaccharides; GAOS: galacturonooligosaccharides; AXWPS: arabinoxylan polysaccharide from wheat flour; AXSPS: glucuronoarabinoxylan polysaccharide from sorghum; AXOS: arabinoxylooligosaccharides; XOS: xylooligosaccharides; FOS: fructooligosaccharides; nd: not determined.

**Table 4.** Fermentative degradation of various polysaccharides and oligosaccharides by several bacteria as determined with HPSEC or HPAEC

| Bacteria | origin | Arabinogalactan | | Arabinan | | (rhamno)galacturonan | | | (arabino)xylan | | | | fructan |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AGPS | AGOS | APS | AOS | RGAPS | RGAOS | GAOS | AXWPS | AXSPS | AXOS | XOS | FOS |
| | blanc | | | | | | | | | | | | |
| Bi. breve | ATCC 15700 | +- | + | - | +- | - | - | nd | - | - | - | nd | + |
| Bi. longum | ATCC 15707 | +- | + | +- | + | - | - | - | + | - | +- | + | + |
| Bi. infantis | ATCC 15697 | - | + | - | +- | - | - | - | - | - | - | - | + |
| Bi. adolescentis | ATCC 15703 | +- | + | +- | +- | - | - | - | - | - | + | + | + |
| C. beijerinckii | human feces | - | nd | +- | +- | - | - | - | - | - | - | + | - |
| C. clostridiiforme | human feces | +- | +- | +- | + | - | - | + | - | - | - | nd | +- |
| C. ramosum | human feces | - | + | +- | - | +- | - | + | - | - | - | - | - |
| C. sporogenes | human feces | - | - | - | - | - | - | - | - | - | - | - | - |
| C. sartagoformum | human feces | +- | - | - | +- | - | - | - | - | - | - | - | - |
| C. perfringens | human feces | - | - | - | - | - | - | - | - | - | - | - | + |
| B. vulgatus | ATCC 8482 | +- | +- | + | +- | +- | + | - | - | +- | +- | + | +- |
| B. ovatus | ATCC 8483 | +- | + | - | - | +- | + | + | + | + | +- | + | +- |
| B. thetaiotaomicron | ATCC 29741 | +- | + | + | - | +- | + | - | - | - | - | - | +- |
| L. casei | Yakult | - | +- | - | - | - | - | - | - | - | - | - | - |
| L. acidophilus | swine feces | +- | + | - | - | - | - | - | - | - | - | + | +- |
| L. fermentum | swine feces | +- | + | + | - | - | - | - | - | - | - | - | +- |
| E. coli | human feces | +- | - | - | +- | - | - | - | - | - | - | - | - |
| K. pneumoniae | human feces | +- | + | - | - | - | - | - | - | - | - | + | +- |

AGPS: arabinogalactan enriched polysaccharide fraction; AGOS: (arabino)galactooligosaccharides; APS: arabinan enriched polysaccharide fraction; AOS: arabinooligosaccharides; RGPS: rhamnogalacturonan enriched polysaccharide fraction; RGAOS: rhamnogalacturonooligosaccharides; GAOS: galacturonooligosaccharides; AXWPS: arabinoxylan polysaccharide from wheat flour; AXSPS: glucuronoarabinoxylan polysaccharide from sorghum; AXOS: arabinoxylooligosaccharides; XOS: xylooligosaccharides; FOS: fructooligosaccharides; + complete degradation, +- partial degradation, - no degradation, nd: not detected

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*Bi. longum* and *B. ovatus* fermented AXWPS completely, as judged from HPSEC (results not shown). HPAEC analysis of the supernatant showed that after 48 h of fermentation of the arabinoxylan polymer by *B. ovatus* the supernatant still contained oligosaccharides.

AXSPS was partially fermented by *B. vulgatus* and this resulted in a small drop in pH. *B. ovatus* fermented the soluble glucuronoarabinoxylan completely over a period of 48 h and no oligomers could be detected in the supernatant.

The linear xylooligosaccharides, xylobiose, -triose and tetraose were fermented by *Bi. longum*, *Bi. adolescentis*, *L. acidophilus*, *Klebsiella pneumoniae* (*K. pneumoniae*), *C. beijerinckii*, *B. vulgatus*, and *B. ovatus*.

The more branched arabinoxylooligosaccharides (Table 2) could only be fermented completely by *Bi. adolescentis*, *Bi. longum* and *B. vulgatus*. *B. ovatus* showed only ß-xylosidase activity toward the xylose residue present at the non-reducing end of the double substituted oligomer 6.1 resulting in the formation of the oligosaccharide 5.1 (Table 2). The other strains tested showed no degradation (Table 4).

### Fructooligosaccharides (FOS)

All *Bifidobacterium* spp. and *Bacteroides* spp. tested and some *Clostridia* spp., *Lactobacillus casei* (*L. casei*) and *K. pneumoniae* showed acidification of the FOS containing media after 48 h of fermentation. Except for *L. casei* and some *Clostridium* spp. all of them were able to ferment the FOS mixture to some extent. *L. acidophilus* and *L. fermentum* fermented only 1-kestose.

## DISCUSSION

The oligosaccharides at present commercially available are produced by extraction from natural plant foods, by acidic or enzymatic hydrolyses of fructans or by transglycosylation starting from sucrose (Hang and Woodams, 1996), lactose (Zarate and Lopez, 1990), maltose (Hayashi et al, 1994), and starch derivatives by using specific bacterial enzymes (Monsan et al, 1989). Here we describe a new class of fermentable oligosaccharides produced by controlled hydrolysis from various naturally occurring plant cell wall polysaccharides. Soy flour, sugar beet pulp, apple, and wheat flour were selected as raw materials for the production of, respectively, (arabino)galacto-, arabino-, rhamnogalacturono-, and arabinoxylooligosaccharides after extraction of the polysaccharide of interest. The oligosaccharides derived from cell wall polysaccharides are structurally different from the oligosaccharides, which are currently commercially available as prebiotics (Playne and Crittenden, 1996). Little research so far has focused on the production of plant cell wall derived oligosaccharides as potential prebiotics, although these types of oligosaccharides might be formed in the colon due to the action of NSP-fermenting bacteria.

All the soluble polysaccharides and oligosaccharides tested in this study were fermented *in vitro* by faecal inocula (data not shown). Depending on the type of poly- and oligosaccharides different strains were able to degrade these substrates. All substrates could be completely fermented by at least one of the tested bacteria.

Within the group of *Bacteroides,* the individual species tested, fermented all or most of the substrates to some extent, showing that *Bacteroides* spp. have a wide variety of glycanases and glycosidases. The rhamnogalacturonooligosaccharides were the only type of oligosaccharides that were selectively fermented by them. The *Bacteroides* spp. are known to degrade xylans containing low levels of arabinose (Salyers et al, 1981; Cooper et al, 1985). In addition, our study showed that highly branched xylans from wheat flour and sorghum and oligosaccharides derived from them were fully fermented by some *Bacteroides* spp. In addition to the type II arabinogalactan (ß1→3, 1→6 arabinogalactans) (Salyers et al, 1981), our study showed that bacteroides were also able to ferment type I arabinogalactan (ß1-4 arabinogalactans) and galactooligosaccharides.

*Clostridium* spp. were also able to ferment most of the substrates to some extent although they showed low activity toward the highly branched xylans and rhamnogalacturans. The fermentation capabilities of intestinal *Clostridium* spp. are not often reported as these species of bacteria are normally not dominant in the colon (Finegold et al, 1983). However several studies showed high saccharolytic activity of *Clostridium* spp. occurring in nature (Henrissat, 1997).

Our studies show that individual species belonging to bifidobacteria are able to ferment (arabino)galactooligosaccharides, arabinooligosaccharides, arabinoxylooligosaccharides, xylooligosaccharides, and fructooligosaccharides but are not able to utilise rhamno- and galacturonooligosaccharides. Bifidobacteria possess high glycosidase activity toward linear substrates such as fructooligosaccharides and galactooligosaccharides. This study shows that bifidobacteria also produce enzymes, which degrade plant cell wall, derived oligosaccharides. Van Laere et al (1997) showed that *Bi. adolescentis* when grown on arabinoxylooligosaccharides produced three different enzymes able to degrade the double substituted arabinoxylooligosaccharides. The arabinoxylan polysaccharides (AXWPS by *Bi. longum*) were also fermented to some extent by some bifidobacteria however, the oligosaccharides derived from the related polysaccharides were generally fermented to a higher extent. Yamada et al (1993) already reported that bifidobacteria fermented arabinoxylooligosaccharides obtained by degradation of wheat bran *in vitro*. Our experiments however provide no information on the growth rate. Further *in vitro* and *in vivo* studies will be needed to evaluate whether these types of oligosaccharides selectively stimulate the growth and/or activity of a selected number of beneficial bacteria.

Fermentation of a specific substrate was not always accompanied with a drop in pH, and this supports the conclusions of Barry et al (1995), that a pH drop should not be used as an index for fermentation. HPAEC and HPSEC are better methods to specifically follow fermentation

of oligo- and polysaccharides, but complete depolymerisation does not always mean full utilisation.

Our experiments give information on the capability of intestinal species of bacteria to utilise the polysaccharides and oligosaccharides. Our studies, however, do not allow predictions about the fermentation *in vivo*, since this will depend on various factors such as the availability of other substrates, growth factors, intestinal pH, actual number of bacteria, and the interactions between the different species of bacteria present.

It can be concluded that the *Bacteroides* spp., predominant intestinal bacteria, are also able to utilise plant cell wall derived oligosaccharides besides their reported activity toward plant polysaccharides. Bifidobacteria are able to utilise the structurally rather complex oligosaccharides derived from plant cell wall polysaccharides in addition to the bifidogenic oligosaccharides such as fructooligosaccharides and xylooligosaccharides, showing their broad range of glycosidases. The degradation of oligosaccharides and polysaccharides by *E. coli* might be important in humans which host high numbers of these organisms in the small intestine. The ability of the lactobacilli and bifidobacteria to ferment specific oligosaccharides and polysaccharides might be important in the development of synbiotics.

## REFERENCES

Ahmed AE, Labavitch JM (1977) A simplified method for accurate determination of cell wall uronide content. J Food Biochem 1: 361-365

Alles MS, Hautvast JGAJ, Nagengast FM, Hartemink R, Van Laere KMJ, Jansen JBMJ (1996) Fate of fructooligosaccharides in the human intestine. Br J Nutr 76: 211-221

Barry J-L, Hoebler C, Macfarlane GT, Macfarlane S, Mathers JC, Reed KA, Mortensen PB, Norgaard I, Rowland IR, Rumney CJ (1995) Estimation of the fermentability of dietary fibre *in vitro*: a European interlaboratory study. Br J Nutr 74: 303-322

Barthomeuf C, Pourrat H (1995) Production of high-content fructo-oligosaccharides by an enzymatic system from *Penicillium rugulosum*. Biotechnol Lett 17: 911-916

Beldman G, Searle van Leeuwen MJF, Ruiter de GA, Siliha HA, Voragen AGJ (1993) Degradation of arabinans [including branched and partly debranched arabinans from sugarbeet pulp] by arabinanases from *Aspergillus aculeatus* and *Aspergillus niger*. Carbohydr Polym 20: 159-168

Buddington RK, Williams CH, Chen SC, Witherly SA (1996) Dietary supplement of neosugar alters the fecal flora and decreases activities of some reductive enzymes in human subjects. Am J Clin Nutr 63: 709-716

Campbell JM, Bauer LL, Fahey GC, Hogarth AJCL, Wolf BW, Hunter DE (1997) Selected fructooligosaccharide (1 kestose nystose and 1(F) beta fructofuranosylnystose) composition of foods and feeds. J Agric Food Chem 45: 3076-3082

Crociani F, Alessandrini A, Mucci MM, Biavati B (1994) Degradation of complex carbohydrates by *Bifidobacterium* spp. Int J Fd Microbiol 24: 199-210

Cooper SW, Pfeiffer DG, Tally FP (1985) Evaluation of xylan fermentation for the identification of *Bacteroides ovatus* and *Bacteroides thetaiotaomicron*. J Clin Microbiol 22: 125-126

Coussement P (1995) Raftilose and raftline: a new generation of dietary fibre. Dtsch Milchwirtsch 46 1060-1062

Dendene K, Guihard L, Nicolas S, Bariou B (1994) Kinetics of lactose isomerisation to lactulose in an alkaline medium. J Chem Technol Biotechnol 61: 37-42

Englyst HN, Cummings JH (1984) Simplified method for the measurement of total non-starch polysaccharides by gas liquid chromatography of constituent sugars of alditol acetates. The Analyst 109: 937-942

Finegold SM, Sutter VL, Mathisen GE (1983) Normal Indigenous Intestinal Flora. In: Hentges D (Ed), Human intestinal flora in Health and Disease. Academic Press: New York 3-31

Garleb KA, Snook JT, Marcon MJ, Wolf BW, Johnson WA (1996) Effect of fructooligosaccharide containing enteral formulas on subjective tolerance factors, serum chemistry profiles and faecal bifidobacteria in healthy adult male subjects. Microbial Ecol Health Disease 9: 279-285

Gibson GR, Roberfroid MB (1995) Dietary modulation of the human colonic microbiota. J Nutr 125: 1401-1412

Graaff de LH, Broeck van den HC, Ooijen van AJJ, Visser J (1994) Regulation of the xylanase-encoding xlnA gene of *Aspergillus tubigensis*. Mol Microbiol 12: 479-490

Gruppen H, Hoffman RA, Kormelink FJM, Voragen AGJ, Kamerling JP, Vliegenthart JFG (1992) Characterisation of $^1$H-NMR spectroscopy of enzymatically derived oligosaccharides form alkali-extractable wheat flour arabinoxylan. Carbohydr Res 233: 45-64

Hang YD, Woodams EE (1996) Optimization of enzymatic production of fructooligosaccharides from sucrose. Lebensm-Wiss Technol 29: 578-580

Hartemink R, Van Laere KMJ, Mertens AKC, Rombouts FM (1996) Fermentation of xyloglucan by intestinal bacteria. Anaerobe 2: 223-230

Hayashi S, Hinotani T, Takasaki Y, Imada K (1994) The enzymatic reaction for the production of panose and isomaltose by glucosyltransferase from *Aureobasidium*. Lett Appl Microbiol 19: 247-248

Henrissat B (1997) A new cellulase family. Mol Microbiol 23: 848-849

Huisman MMH, Schols HA, Voragen AGJ (1998) Cell wall polysaccharides from soybean (*Glycine max*) meal:Isolation and characterisation. Carbohydr Polym 37: 87-95

Ito M, Deguchi Y, Matsumoto K, Kimura M, Onodera N, Yajima T (1993) Influence of galactooligosaccharides on the human fecal microflora J Nutr Sc Vitaminol 39: 635-640

Knorr D (1998) Technology aspects related to microorganisms in functional foods. Trends Food Sc Technol 9: 295-306

Monsan P, Paul F, Remaud M, Lopez A (1989) Novel enzymatic synthesis of oligosaccharides. Food Biotechnol 3: 11-29

Macfarlane GT, Cummings JH (1991) The colonic flora fermentation and large bowel digestive function. In: Philips SF, Pemberton JH, Shorter RG (Eds) The large intestine: physiology, pathophysiology and disease: 51-92 Raven Press: New York

Playne MJ, Crittenden R (1996) Commercially available oligosaccharides. Bull Int Dairy Fed 313: 10-22

Salyers AA, Gherardini F, O'Brien M (1981) Utilization of xylan by two species of human colonic *Bacteroides.* Appl Environ Microbiol 41: 1065-1068

Schols HA, Posthumus MA, Voragen AGJ (1990) Structural features of hairy regions of pectins isolated from apple juice produced by the liquefaction process. Carbohydr Res 206: 117-129

Schols HA, Voragen AGJ, Colquhoun IJ (1994) Isolation and characterization of rhamnogalacturonan oligomers liberated during degradation of pectic hairy regions by rhamnogalacturonase. Carbohydr Res 256: 97-111

Thurl S, Mullerwerner B, Sawatzki G (1996) Quantification of individual oligosaccharide compounds from human milk using high pH anion-exchange chromatography. Anal Biochem 235: 202-206

Van Laere KMJ, Beldman G, Voragen AGJ (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

Voragen AGJ (1998) Technological aspects of functional food-related carbohydrates. Trends Food Sc Technol 9: 328-335

Verbruggen MA, Beldman G, Voragen AGJ (1995) The selective extraction of glucuronoarabinoxylans from sorghum endosperm cell walls using barium and potassium hydroxide solutions. J Cereal Sci 21: 271-282

Vercellotti JR, Salyers AA, Bullard WS, Wilkins TD (1977) Breakdown of mucin and plant polysaccharides in the human colon. Can J Biochem 55: 1190-1196

Versteeg C (1979) Pectinesterases from the orange fruit- their purification general characteristics and juice cloud destablizing properties. PhD thesis Wageningen Agricultural University The Netherlands

Yamada H, Itoh K, Morishita Y, Taniguchi H (1993) Structure and properties of oligosaccharides from wheat bran. Cereal Foods World 38: 490-492

Yun JW, Kim DH Kim BW, Song KS (1997) Comparison of sugar compositions between inulo- and fructo-oligosaccharides produced by different enzyme forms. Biotechnol Lett 19: 553-556

Zarate S, Lopez-Leiva MH (1990) Oligosaccharide formation during enzymatic lactose hydrolysis: a literature review. J Food Prot 53: 262-26

# 3

## *A new arabinofuranohydrolase from Bifidobacterium adolescentis able to remove arabinofuranosyl residues from double-substituted xylose units in arabinoxylan*

An arabinofuranohydrolase (AXH-d3) was purified from a cell-free extract of *Bifidobacterium adolescentis* DSM 20083. The enzyme had a molecular weight of approximately 100 kDa as determined by size exclusion. It displayed maximum activity at pH 6 and 30°C. Using an arabinoxylan derived oligosaccharide containing double-substituted xylopyranosyl residues, it was established that the enzyme specifically released terminal arabinofuranosyl residues linked to C-3 of double-substituted xylopyranosyl residues. Additionally, this arabinofuranohydrolase released arabinofuranosyl groups from wheat flour arabinoxylan polymer but showed no activity towards *p*-nitrophenyl-α-L-arabinofuranoside or towards sugar beet arabinan, soy arabinogalactan, arabinooligosaccharides and arabinogalactooligosaccharides.

## INTRODUCTION

Xylans are widely distributed types of hemicelluloses in plant cell walls. These hetero-polysaccharides consist of a ß-D-(1→4)-linked xylopyranoside backbone and can be substituted with α-L-arabinofuranose, ß-D-glucopyranosyl uronic acid, or its 4-O-methyl derivative, and acetyl groups. The complete degradation of substituted xylans involves the action of several hydrolytic enzymes: endo-xylanases, ß-xylosidases, glucuronoxylan hydrolases, and enzymes capable of hydrolysing substituents from the xylan backbone such as α-L-arabinofuranosidases, acetyl and feruloyl esterases, uronidases and glucosidases (Coughlan and Hazlewood, 1993).

α-L-Arabinofuranosidases can hydrolyse arabinofuranosyl linkages from various hemicelluloses such as arabinans, (arabino)xylans, (arabino)galactans and arabinose-substituted xyloglucans. Also, oligosaccharides containing arabinofuranosyl-groups and synthetic substrates such as *p*-nitrophenyl-α-L-arabinofuranoside (*p*-NP-ara*f*) are mentioned as substrates for arabinosidases (Kaji,1984). Two types of α-L-arabinofuranosidases and an arabinoxylan arabinofuranohydrolase (AXH) able to cleave of arabinofuranosyl groups from arabinoxylans have been identified in *Aspergillus sp.*. α-L-arabinofuranosidase A is active towards arabinan oligosaccharides and arabinoxylan oligosaccharides as well as *p*-NP-ara*f*. α-L-arabinofuranosidase B is active towards polymeric arabinan, arabinoxylan, arabinogalactan and oligosaccharides containing arabinofuranosyl groups, as well as *p*-NP-ara*f* (Rombouts et al, 1988). Arabinoxylan arabinofuranohydrolase (AXH) shows activity towards C-3 linked arabinofuranosyl substituents present at single-substituted xylopyranosyl residues in arabinoxylans (Kormelink et al, 1991).

Until now no enzyme was found which was active towards either C-2 or C-3 linked arabinofuranosyl substituents from double-substituted xylopyranosyl residues.

In preliminary experiments using *Bi. adolescentis DSM 20083* and enzyme extracts thereof, we observed fermentative and enzymatic degradation of arabinoxylan fragments containing double-substituted xylose residues. Here we report on the identification of one of the responsible arabinofuranohydrolases (AH) which hydrolyses arabinofuranosyl linkages at the C-3 of double-substituted xylopyranosyl residues.

## MATERIALS AND METHODS

### Substrates

Wheat flour arabinoxylan was obtained from Megazyme (International Ireland Ltd, Wicklow Ireland), soy arabinogalactan was a gift from Novo Nordisk Ferment AG (Dittingen, Switzerland), linear sugar beet arabinan was a gift from British Sugar (Peterborough, UK), and *p*-NP-ara*f* was obtained from Sigma (St-Louis, MI).

A mixture of arabinoxylan oligosaccharides and the arabinoxylan oligosaccharide 6.1 (Fig 1) (Gruppen et al, 1994) was obtained by degradation of arabinoxylan from wheat flour with an endo-xylanase from *Aspergillus tubigensis* (Graaf et al, 1994). The digest was fractionated using two columns (600x26mm) in series packed with Fractogel TSK HW-40(S) (25-40 μm, Merck, Darmstadt, Germany) (Verbruggen, 1996). The fractions were monitored for purity using High-Performance Anion-Exchange Chromatography (HPAEC) and compared to an oligosaccharide 6.1 standard (Gruppen et al, 1992). The degradation of the oligosaccharide 6.1 by AH was followed by HPAEC on a Dionex Bio-LC system (Dionex, Sunnyvale, CA) according to the same authors. The separation method was slightly modified by using a linear gradient of 0 - 0.2 M sodium acetate in 0.1 M sodium hydroxide for 0-30 min.
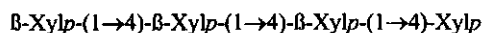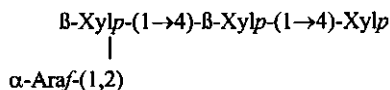
## Production and purification of enzyme

The arabinofuranohydrolase (AH) was isolated from a cell free extract of *Bi. adolescentis* DSM 20083 grown in 5 L of M17 broth (OXOID, Unipath LTD, Basingstoke, Hampshire, UK) supplemented with 0.5 % (w/v) arabinoxylan oligosaccharides (conditions: batch, statically, anaerobically, 37°C). When the culture reached the stationary phase of growth, the cells were harvested by centrifugation (10,000 * g, 10 min, 4°C). This step and all other procedures were carried out under aerobic conditions. Cells were washed once in 20 mM potassium phosphate buffer (pH=6.5) and then resuspended in the same buffer. Cells were disrupted by sonic treatment (15 min, duty cycle 30%) with a sonifier cell disrupter. During sonic treatment, the tube containing the cell suspension was kept on ice. Subsequently the suspension was centrifuged at 30,000 * g for 60 min to remove undisrupted cells and the cell-debris. The supernatant was used for purification. Enzyme purification was carried out on a hydroxyapatite column (2.2 by 18 cm) (Bio-Rad Laboratories, Hercules, CA), MonoQ 5/5 column (Pharmacia-LKB Biotechnology, Uppsala, Sweden) and a S-200 column (1.6 by 100 cm) (Pharmacia-LKB Biotechnology, Uppsala, Sweden). The S-200 column was calibrated using the following standards: Thyroglobulin (669 kDa), Catalase (440 kDa), Aldolase (158 kDa), Ovalbumin Serum Albumin (67 kDa), Ribonuclease (13,7 kDa) (Pharmacia-LKB Biotechnology, Uppsala, Sweden). The AH-activity was assayed towards arabinoxylan oligosaccharide 6.1 (Fig 1; see further) using HPAEC.

## Characterisation of reaction products

The degradation products obtained after incubation of the oligosaccharide 6.1 with AH were produced on a preparative scale and purified using gel permeation chromatography (BioGel P-2; Bio-Rad Laboratories, Hercules, CA). Oligosaccharide 6.1 and the oligomeric degradation product were lyophilised twice from 99.9 % $D_2O$ and once from 99.96 % $D_2O$ (Cambridge Isotope laboratories, Andover, MA). The components were dissolved in 0.7 ml 99.96 $D_2O$ in a 5 mm NMR tube, equilibrated at 30°C. The $^1H$-NMR spectra were recorded at 30°C in a Brüker DPX-400 spectrometer operated at 400 MHz and equipped with a 5 mm $^1H/^{13}C$ probe

(acquisition time 4 s; number of scans 360). Spectra were referenced to the residual HDO resonance at 4.74 ppm. The activity towards the different substrates was measured *in* 50 mM phosphate buffer pH 6 at 30°C. Side activities, pH and temperature optimum were determined according to Mutter et al (1994).

ß-Xyl*p*-(1→4)-ß-Xyl*p*-(1→4)-ß-Xyl*p*-(1→4)-Xyl*p*

**(X₄)**

ß-Xyl*p*-(1→4)-ß-Xyl*p*-(1→4)-Xyl*p*
$\quad\quad$ |
α-Ara*f*-(1,2)

**(RV-41)**

ß-Xyl*p*-(1→4)-ß-Xyl*p*-(1→4)-Xyl*p*
$\quad$ / |
α-Ara*f*-(1,3) |
$\quad$ α-Ara*f*-(1,2)

**(5.1)**

ß-Xyl*p*-(1→4)-ß-Xyl*p*-(1→4)-ß-Xyl*p*-(1→4)-Xyl*p*
$\quad\quad$ /
$\quad$ α-Ara*f*-(1,3)

**(5.4), AX31**

ß-Xyl*p*-(1→4)-ß-Xyl*p*-(1→4)-ß-Xyl*p*-(1→4)-Xyl*p*
$\quad\quad$ |
$\quad$ α-Ara*f*-(1,2)

**(5.5)**

ß-Xyl*p*-(1→4)-ß-Xyl*p*-(1→4)-ß-Xyl*p*-(1→4)-Xyl*p*
$\quad\quad$ / |
$\quad$ α-Ara*f*-(1,3) |
$\quad\quad$ α-Ara*f*-(1,2)

**(6.1), AX33**

ß-Xyl*p*-(1→4)-ß-Xyl*p*-(1→4)-ß-Xyl*p*-(1→4)-ß-Xyl*p*-(1→4)-Xyl*p*
$\quad\quad$ / |
$\quad$ α-Ara*f*-(1,3) |
$\quad\quad$ α-Ara*f*-(1,2)
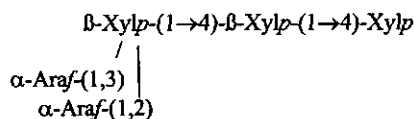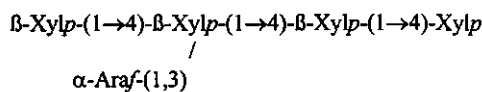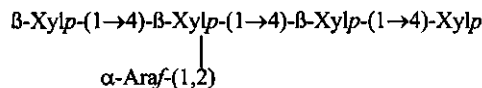
**(7.2)**

**Figure 1.** Structures of arabinoxylan oligosaccharides. Nomenclature according to Gruppen et al (1992) (6.1; 5.4; 5.1; 7.2), Hoffmann et al (1991) (AX33; AX31) or Viëtor et al (1994) (RV-41).

## RESULTS

Preliminary experiments revealed that double-substituted oligosaccharides derived from arabinoxylan were totally fermented by *Bi. adolescentis*. This indicated that during growth on these oligosaccharides *Bi. adolescentis* produced enzymes able to degrade these oligosaccharides. For production of the responsible enzymes, and subsequent purification, *Bi. adolescentis* was grown on a mixture of double-substituted arabinoxylan oligosaccharides and the culture liquid as well as the cells was assayed for the presence of arabinofuranose releasing enzymes. No activity was found in the culture liquid, however, the cell-free extract, obtained after ultrasonic treatment of the cells followed by centrifugation, contained enzymes which were able to split off both arabinoses from the oligosaccharide 6.1 (data not show).

Using sequentially adsorption chromatography, anion exchange chromatography and size exclusion, an arabinofuranohydrolase which splits of arabinofuranosyl groups from double-substituted xylose residues, was obtained pure and without detectable side activities. The purified AH showed maximum activity at pH 6 and 30°C. The molecular weight, as determined by size exclusion, appeared to be 100 kDa.
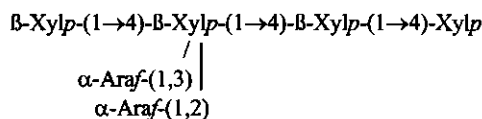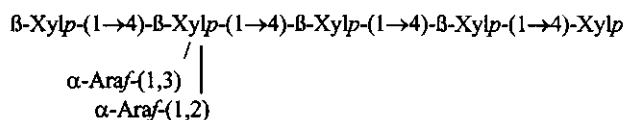
AH could hydrolyse arabinofuranosyl linkages in arabinoxylan from wheat flour even when this substrate was extensively pretreated with AXH. The mode of action of AH was determined at pH 6 and 30°C using the oligosaccharide 6.1. Degradation of the oligosaccharide 6.1 with AH resulted in the formation of arabinose, undegraded oligosaccharide 6.1 and a product with unknown structure (Fig 2).
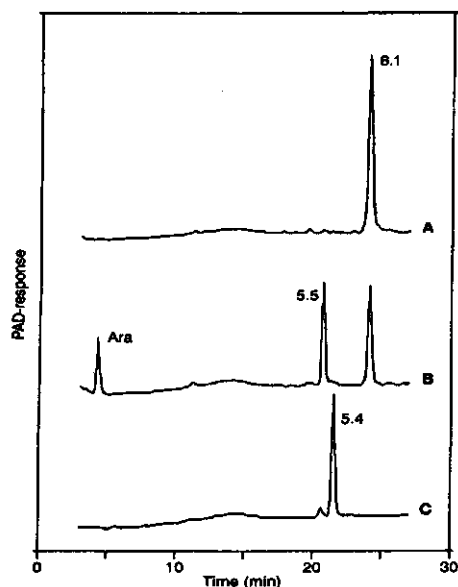


**Figure 2.** HPAEC analysis of the reaction products obtained after incubation of the oligosaccharide 6.1 with arabinofuranohydrolase (AH). A. untreated oligosaccharide 6.1; B. oligosaccharide 6.1 treated with AH; C. standard oligosaccharide 5.4

Essentially, the removal of arabinose from the oligosaccharide 6.1 could lead to the formation of three different products namely the oligosaccharides $X_4$, 5.4 and 5.5 (Fig 1).

Comparison of the retention times of the oligosaccharide $X_4$ (15 min) and the degradation product (20.7 min) in HPAEC analysis excluded the possibility that the degradation product was the oligosaccharide $X_4$. This shows that only one arabinose had been removed from the oligosaccharide 6.1. The retention time of the degradation product (20.7 min) was slightly different from that of the oligosaccharide 5.4 (21.5 min) indicating that the product formed could be the oligosaccharide 5.5. The product eluting at 20.7 min was produced on a preparative scale and purified using size-exclusion on BioGel P2. Its structure was determined by $^1$H-NMR.

The relevant part of the $^1$H-NMR spectrum of the untreated oligosaccharide 6.1 is shown in Fig 3a. The chemical shift data matched exactly those of the oligosaccharide AX-33 and thus the structure could be confirmed as being the oligosaccharide 6.1 (Hoffmann et al, 1991). The signals at 5.274 and 5.224 ppm were assigned to the anomeric protons of the terminal arabinofuranosyl residues linked to C-3 and C-2, respectively, of double-substituted xylopyransoyl residues.
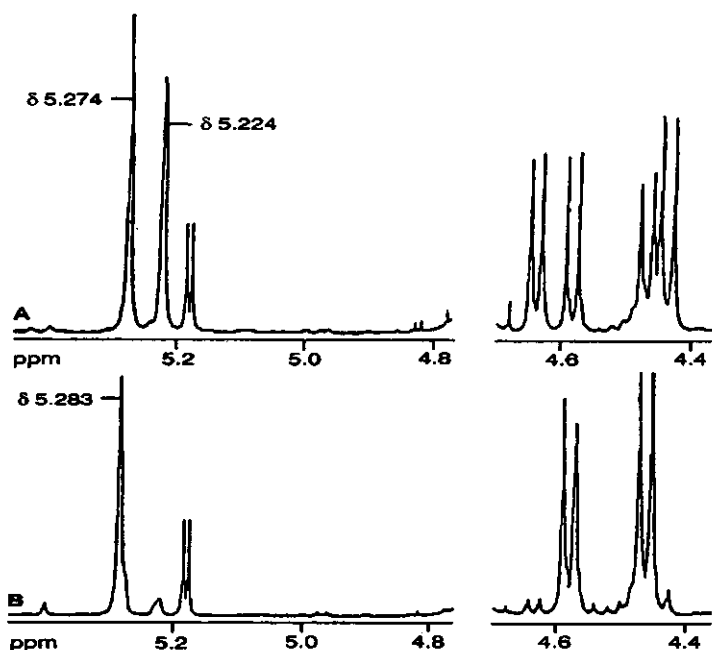


**Figure 3.** $^1$H-Nuclear magnetic resonance (NMR) spectrum of 6.1 (a) and 5.5 (b); δ, chemical shift. The signal at 5.274 and 5.224 ppm were assigned to the anomeric protons of the terminal arabinofuranosyl residues linked to C-3 and C-2 respectively, of double-substituted xylopyranosyl residues. The signal at 5.283 ppm was assigned to the anomeric proton of the terminal arabinofuranosyl residues linked to C-2 of single-substituted xylopyranosyl residues.

Hoffmann et al (1991) showed that the anomeric signal of terminal arabinofuranosyl residues linked to C-3 of single-substituted xylopyranosyl groups in the oligosaccharide 5.4 shifted downfield to 5.397 ppm compared to the signal of terminal arabinofuranosyl residues linked to C-3 of double-substituted xylopyranosyl groups in the oligosaccharide 6.1. Viëtor et al (1994) observed that the anomeric signal of terminal arabinofuranosyl linked to C-2 of single-substituted xylopyranosyl groups of the oligosaccharide RV-41 shifts downfield to 5.28 ppm compared to the signal of terminal arabinofuranosyl residues linked to the C-2 of double-substituted xylopyranosyl residues in the oligosaccharide 5.1.

Fig 3b shows the $^1$H-NMR spectrum of the degradation product. The chemical shift data obtained for the degradation product did not match with the oligosaccharide AX-31 (5.4) as described by Hoffmann et al (1991). The signal at 5.280 ppm was assigned to the terminal arabinofuranosyl residues linked to C-2 of the single-substituted xylopyranosyl residues (Viëtor et al, 1994) and the structure was assigned as being the oligosaccharide 5.5 (Fig 1).

During the purification of AH from *Bi. adolescentis* it appeared that different arabinofuranohydrolases were present in the cell free extract. Also another new arabinofuranohydrolase able to remove arabinose from the C-2 position of single-substituted xylose residues, was present in the mixture. This enzyme is described in Van Laere et al (1999).

The AH described in the present communication also released arabinofuranosyl groups from other double-substituted arabinoxylan oligosaccharides such as the oligosaccharides 5.1 and 7.2 (Fig 1) but the enzyme showed no activity towards the single-substituted oligosaccharides 5.4 or 5.5 (results not shown). AH did not release any arabinose from soy arabinogalactan, sugar beet arabinan or oligosaccharides derived from these substrates and showed no activity towards *p*-NP-ara*f*.

## DISCUSSION

Untill now no report has been published on the presence of arabinosidases or arabinofuranohydrolases in enzyme extracts of *Bi. adolescentis* DSM 20083. Fermentation experiments revealed the complete degradation of double-substituted arabinoxylan oligosaccharides by *Bi. adolescentis*. The cell-free extract contained enzymes to split of both arabinoses from the oligosaccharide 6.1. Similar activity has been observed for a fungal preparation derived from *Trichoderma reesei* (not shown, to be published, K. Rakasi, 1996, research at our department). AH could hydrolyse arabinofuranosyl linkages in arabinoxylan from wheat flour even when this substrate was extensively pretreated with AXH, which showed that AH is able to split off arabinofuranosyl groups from arabinoxylans, other than those removed by AXH.

Using HPAEC and $^1$H-NMR-spectroscopy the product formed after incubation of the oligosaccharide 6.1 with AH could be assigned as the oligosaccharide 5.5. These results showed that AH was able to release terminal arabinofuranosyl residues linked to C-3 of double-substituted xylopyranosyl residues in the oligosaccharide 6.1. AH also splits off arabinose residues from the oligosaccharide 7.2 and 5.1, but showed no activity towards

single-substituted oligosaccharides 5.4 or 5.5. AH did not release any arabinose from soy arabinogalactan, sugar beet arabinan or oligosaccharides derived from these substrates and showed no activity towards *p*-NP-araf. This showed that AH differed from the *A. niger* and *Streptomyces purpurascens* type of α-L-arabinofuranosidase which were both active towards *p*-NP-araf and AH could not be classified accordingly (Kaji, 1984).

From these results it can be concluded that *Bi. adolescentis* produces a new enzyme specific for arabinoxylans, able to release arabinofuranosyl groups from the C-3 position of double-substituted xylopyranosyl residues in the xylan backbone. In Addition to AXH from *Aspergillus sp.*, we describe here an enzyme which is specific for arabinoxylan and which can also be denoted as an arabinoxylan arabinofuranohydrolase. Since AXH is able to remove arabinofuranosyl residues from single-substituted xylopyranosyl residues only, it seems to be necessary to update the nomenclature of this type of enzymes. AXH could be denoted as AXH-m (mono substituted xylopyranosyl residues) while the enzyme describe in this paper (AH) could be denoted as AXH-d3 indicating that the enzyme is specific for arabinofuranosyl groups linked at the C-3 position of double-substituted xylopyranosyl residues. Thus, AXH-d3 plays a key role in the total degradation of arabinoxylans.

## ACKNOWLEDGMENTS

## REFERENCES

Coughlan MP & Hazlewood GP (1993) ß-1,4-D-Xylan-degrading enzyme systems: biochemistry, molecular biology and applications. Biotechnol Appl Biochem 17: 259-289

de Graaf LH, van de Broek HC, van Ooijen AJJ, Visser J (1994) Regulation of the xylanase-encoding *xlnA* gene of *Aspergillus tubigensis*. Mol Microbiol 12: 479-490

Gruppen H, Hoffmann RA, Kormelink FJM, Voragen AGJ, Kamerling JP, and JFG Vliegenthart (1992) Characterisation by ^{1}H-NMR spectroscopy of enzymically-derived oligosaccharides from alkali-extractable wheat flour arabinoxylan. Carbohydr Res 233: 45-64

Hoffmann RA, Leeflang BR, de Barse MMJ, Kamerling JP and Vliegenhart JFG (1991). Characterisation by ^{1}H-NMR spectroscopy of oligosaccharides, derived from arabinoxylans of white endosperm of wheat, that contain the elements →4[α-L-Araf-(1→3)]-ß-D-Xylp-(1→or→4)[α-L-Araf-(1→2)][α-L-Araf-(1→3)]-ß-D-Xylp-(1→. Carbohydr Res 221: 63-81

Kaji A (1984) L-Arabinosidases. Adv Carbohydr Chem Biochem 42: 383-394

Kormelink FJM, Searle-Van Leeuwen MJF, Wood TM & Voragen AGJ (1991) Purification and characterisation of an (1-4)-ß-D-arabinoxylan arabinofuranohydrolase from *Aspergillus awamori*. Appl Microbiol Biotechnol 35: 753-758

Mutter M, Beldman G, Schols HA, Voragen AGJ (1994) Rhamnogalacturonan α-L-Rhamnopyranohydrolase: A novel enzyme specific for the terminal nonreducing rhamnosyl unit in rhamnogalacturonan regions in pectin. Plant Physiol 106: 241-250

Rombouts FM, Voragen AGJ, Searle- Van Leeuwen MJF, Geraeds CCJM, Schols HA and Pilnik W (1988) The arabinanases of *Aspergillus niger*- Purification and characterisation of two α-L-arabinofuranosidases and an endo-1→5-α-L-Arabinanase. Carbohydr Polym 9: 25-47

Van Laere KMJ, Voragen CHL, Kroef T, Van den Broek LAM, Beldman G, Voragen AGJ (1999) Purification and mode of action of two different arabinoxylan arabinofuranohydrolases from *Bifidobacterium adolescentis* DSM 20083 Appl Microbiol Biotechnol 51: 606-613 (Chapter 4)

Verbruggen MA (1996) Glucuronoarabinoxylans from sorghum grain. PhD thesis, Wageningen Agricultural University, The Netherlands

Viëtor RJ, Hoffman RA, Angelino SAGF, Voragen AGJ, Kamerling JP, Vliegenthart JFG (1994) Structures of small oligomers liberated from barley arabinoxylans by endoxylanase from *Aspergillus awamori*. Carbohydr Res 254: 245-255

# 4

# Purification and mode of action of two different arabinoxylan arabinofuranohydrolases from Bifidobacterium adolescentis DSM 20083

Two novel arabinofuranohydrolases (AXH-d3 and AXH-m23) were purified from *Bifidobacterium adolescentis* DSM 20083. Both enzymes were induced upon growth of *Bi. adolescentis* on xylose and arabinoxylan-derived oligosaccharides. They were only active towards arabinoxylans and therefore denoted as arabinoxylan arabinofuranohydrolases. Their optimal activity was at pH 6 and 30-40°C.

They were very specific in their mode of action and were clearly different from AXH-m from *Aspergillus awamori*. AXH-m23 released only arabinofuranosyl groups which were linked to the C-2 or C-3 position of single-substituted xylose residues in arabinoxylan oligomers. AXH-d3 hydrolysed C-3 linked arabinofuranosyl residues of double-substituted xylopyranosyl residues of arabinoxylans and arabinoxylan-derived oligosaccharides. No activity was observed with C-2 linked arabinofuranosyl residues of these double-substituted xylopyranosyl residues, or against C-2 and C-3 linked arabinofuranosyl residues of single-substituted xylopyranosyl residues. The combination of AXH-d3 and AXH-m showed low debranching activity against highly substituted glucurono-arabinoxylans. However, arabinoxylan from wheat flour was debranched almost completely.

## INTRODUCTION

Non-digestible oligosaccharides (NDOs), such as fructooligosaccharides are regarded as substrates which selectively stimulate the growth and/or activity of one or a selection of beneficial bacteria (Gibson & Roberfroid, 1995) such as bifidobacteria. Bifidobacteria posses a range of glycosidases which are active towards oligosaccharides originating from different sources such as arabinogalactan, arabinan and arabinoxylans. Previous studies showed that *Bi. adolescentis* is one of the few bacteria which is able to ferment double-substituted xylooligosaccharides having arabinofuranosyl residues (Van Laere 1997a), and that it is producing very specific enzymes to do this. Van Laere et al (1997b) showed that *Bi. adolescentis* produces at least one specific arabinoxylan arabinofuranohydrolase which is involved in the degradation of these oligosaccharides. Specific arabinoxylan arabinofuranohydrolases have also been isolated from *Aspergillus awamori* (Kormelink, 1991b), *Pseudomonas fluorescens* (Kellet et al, 1990), wheat (Beldman et al, 1996), and *Trichoderma reesei* (unpublished work). Although these enzymes are very specific for arabinoxylans, their mode of action on arabinoxylans and arabinoxylan-derived oligosaccharides is different. While the arabinoxylan arabinofuranohydrolases from *A. awamori*, *P. fluorescens* and wheat are mainly active towards arabinofuranosyl residues of single-substituted xylopyranosyl residue, the one of *Bi. adolescentis* and *T. reesei* are the first arabinoxylan arabinofuranohydrolases reported active towards arabinofuranosyl residues of double-substituted xylopyranosyl residues.

The discovery of such highly specific enzymes raises the possibility that other AXHs with different specificity may also exist. Therefor we wanted to study in detail the mode of action of two arabinoxylan arabinofuranohydrolases from *Bi. adolescentis* and to compare this with the mode of action of AXH from *A. awamori*.

## MATERIALS AND METHODS

### Substrates

Wheat flour arabinoxylan, with an Ara/Xyl ratio of 0.43 was obtained from Megazyme (International Ireland Ltd, Wicklow, Ireland) and *p*-nitrophenyl-arabinofuranose (*p*-NP-ara*f*) from Sigma (St-Louis, Mo.). Soy arabinogalactan and linear sugar beet arabinan were a gift from Novo Nordisk Ferment AG (Dittingen, Switzerland) and British Sugar (Peterborough, UK) respectively.

Arabinoxylan carrying single substitutions of arabinose at either the C-2 and C-3 position of xylose residues was obtained by treating wheat flour arabinoxylan with AXH-d3 (50 mM piperazine pH 6, 40°C). Incubating this single-substituted arabinoxylan with endo-xylanase I resulted in C-2 and C3 single-substituted arabinoxylan oligosaccharides.

(Glucurono)-arabinoxylan was obtained according to Schooneveld-Bergmans et al (1999).
A mixture of arabinoxylan-derived oligosaccharides, including the oligosaccharides 7.2, 6.1, 5.1, xylotriose, and xylobiose (Table 3; nomenclature according to Gruppen et al, 1992), were obtained by degrading arabinoxylan from wheat flour with an endo-xylanase from *A. tubigensis* (Graaf de et al, 1994). The digest was fractionated using two columns (600x26mm) in series packed with Fractogel TSK HW-40(S) (25-40 µm, Merck, Darmstadt, Germany; Verbruggen, 1996. The oligosaccharide 5.5 was produced according to Van Laere et al (1997b).

## Enzymes

ß-D-(1,4)- Arabinoxylan arabinofuranohydrolase (AXH-m) and endo ß-D-(1,4)- xylanase I (endo-xylanase I) were purified from *A. awamori* CMI 142717 (Commonwealth Mycological Institute) as described by Kormelink et al (1991, 1993a). α-L-Arabinofuranosidase A and B were purified from *A. niger* according to Rombouts et al (1988).

## General growth conditions and induction of arabinofuranohydrolases

*Bi. adolescentis* was grown in M17 broth (OXOID, Unipath LTD, Basingstoke, Hampshire, UK) supplemented with 0.5% (w/v) of various carbohydrates [glucose, arabinose, xylose, xylobiose, xylotriose, the oligosaccharides 5.1, 6.1, 7.2 (Table 3), or arabinoxylan polymer from wheat flour] (conditions: batch, statically, anaerobically, 37°C). After growth optical density (605 nm) and pH were measured. The enzyme extracts were prepared as described by Van Laere et al (1997b).

## Production and purification of AXH-m23 and AXH-d3

*Bi. adolescentis* was cultured with 0.5% (w/v) xylose or arabinoxylan oligosaccharides as described above. The purification steps of the enzyme extract involved Bio-Gel HTP hydroxylapatite (Bio-Rad Laboratories, Hercules, CA), Q-Sepharose, Mono Q HR5/5, and Sephacryl S-200 HR 16/60. The three latter columns were from Pharmacia LKB Biotechnology, Uppsala, Sweden. Purification procedures were carried out aerobically at ambient conditions. Fractions were analysed for protein content by $A_{280}$ or the Sedmak method (Sedmak and Grossberg, 1977) with BSA as standard. Native gel electrophoresis and silver staining were carried out with a PhastSystem (Pharmacia LK Biotechnology, Uppsala, Sweden) using an 8 to 25% polyacrylamide gel.

## Enzyme assays

Enzyme activity was measured in 50 mM piperazine buffer pH 6 or 25 mM phosphate buffer pH 7 at 40°C containing 0.05% (w/v) of respectively the oligosaccharide 6.1 or 5.5. The reaction was stopped by heating for 10 min at 100°C. The activity of AXH-d3 and AXH-m23

was measured by High-Performance Anion-Exchange chromatography (HPAEC) as a decrease of the relative area of respectively the oligosaccharide 6.1 and 5.5.

## Modification, degradation, and characterisation of arabinoxylan and arabinoxylan-derived oligosaccharides

Aliquots of arabinoxylan (0.125% w/v), AXH-d3 treated arabinoxylan, and (glucurono)-arabinoxylans were treated with AXH-m, (50 mM acetate buffer pH 4.8, 50°C), AXH-d3, and AXH-m23 (50 mM piperazine buffer pH 6, 40°C) for 24 h and 48 h. The arabinose release was monitored using HPAEC and was compared to the total amount of arabinose present in the arabinoxylan polymer as determined hydrolysis with trifluoroacetic acid. To examine the mode of action of AXH-d3 and AXH-m on wheat arabinoxylan by ^{1}H-NMR, the untreated and treated arabinoxylan was dialysed and freeze-dried in $D_2O$. When insoluble aggregates were formed, the arabinoxylan was slightly treated with endo-xylanase I. Arabinoxylan-derived oligosaccharides, C-2 and C-3 single-substituted arabinoxylan oligosaccharides, and some purified oligosaccharides (6.1, 5.5, 5.4, 5.1, 6.1, 8.1, 9.1, and 6.3) were incubated with AXH-m, (50 mM sodium acetate buffer pH 4.8, 50°C), AXH-d3, or AXH-m23 (50 mM piperazine buffer pH 6, 40°C) for 1h, 24h, or 48h. All the reactions were terminated by boiling for 10 min.

## Analytical measurements

^{1}H-NMR was performed as described by Van Laere et al (1997b). Enzyme treated and untreated arabinoxylan (1.5 mg) were dialysed and then lyophilised from 99.9% $D_2O$ (Cambridge Isotope Laboratories, Andover, MA). The polysaccharides were dissolved in 0.7 ml 99.96 % $D_2O$ in a 5 mm NMR tube and equilibrated at 30°C.

Arabinoxylooligosaccharides derived from alkali-extractable wheat flour arabinoxylans by endo-xylanase I incubation, were used as standards for the identification of some oligosaccharides using HPAEC (Gruppen et al, 1992).

Changes in molecular weight distributions of arabinoxylans treated by enzymes were determined by HPSEC, using three Bio-Gel TSK columns in series as described elsewhere (Bergmans et al, 1996).

Total arabinose content of different arabinoxylan fractions was determined by hydrolyses with 2M trifluoroacetic acid (TFA) for 1 h at 120°C. Subsequently the samples were cooled to room temperature and TFA was evaporated with a stream of dried air at 40°C. The residues were dissolved in 0.2 ml water and analysed by HPAEC. The amount of arabinose formed was compared to an arabinose standard.

## RESULTS

### Induction of arabinofuranohydrolases in *Bi. adolescentis* DSM 20083

*Bi. adolescentis* produced enzymes able to degrade arabinoxylan-derived oligosaccharides when the bacteria were cultured on this type of oligosaccharides. The double-substituted arabinoxylan oligosaccharide 6.1 was degraded completely towards arabinose and xylose by the enzyme-extract. A new arabinoxylan arabinofuranohydrolase purified from *Bi. adolescentis* (AXH-d3) releases the $(1\rightarrow3)$-$\alpha$-L-arabinofuranosyl residues of the double-substituted xylopyranosyl residue present in the oligosaccharide 6.1 which resulted in the formation of the oligosaccharide 5.5 (Table 3) (Van Laere et al, 1997b). AXH-d3 showed no activity towards the oligosaccharide 5.5. Using the enzyme-extract from *Bi. adolescentis* the oligosaccharide 5.5 was further degraded, which showed that another arabinofuranohydrolase was present in the enzyme-extract able to release the $(1\rightarrow2)$-$\alpha$-L-arabinofuranosyl residues of the single-substituted xylopyranosyl residue present in the oligosaccharide 5.5 (AXH-m23).

In order to establish which components in the oligosaccharide mixture were responsible for the induction of the arabinofuranohydrolase activity, enzyme production and growth on the individual components were studied. *Bi. adolescentis* was cultured on a medium containing different substrates (no carbon sources, glucose, arabinose, xylose, xylobiose, xylotriose, and the oligosaccharides 5.1, 6.1, 7,2). An absorbance of 1.0 after 24 h incubation was reached on all substrates except when there was no carbon source present (A= 0.2). The highest activity (2 U/mg) was obtained by culturing on the oligosaccharides 6.1, 5.1, and 7.2 as a carbon source. AXH-d3 was also induced by growing on xylose, xylobiose, and xylotriose (1 U/mg). No AXH-d3 activity was observed using the other carbon sources even after prolonged incubation times. AXH-m23 was present in the same enzyme-extracts as AXH-d3.

### Purification of the arabinofuranohydrolase AXH-d3 and AXH-m23

Cell extracts from *Bi. adolescentis* were fractionated using Q-Sepharose, Bio-Gel HTP hydroxyapatite, Mono Q HR5/5, and Sephacryl S-200 HR 16/60. Details are given in the purification scheme (Table 1). The cell extracts were initially fractionated on a Q-Sepharose column. The fractions with the highest activity towards the oligosaccharide 6.1 and 5.5 were pooled, dialysed against 20 mM piperazine buffer pH 6 and applied again onto the Q-Sepharose. Fractions with the highest AXH-d3 and AXH-m23 activity were pooled, dialysed against 20 mM phosphate buffer pH 7, and applied onto an hydroxyapatite column. This purification step resulted in the separation between AXH-m23 and AXH-d3 (not shown). The fractions eluting in 50-60 mM phosphate buffer (AXH-d3) and in 100-120 mM phosphate buffer (AXH-m23) were pooled separately and concentrated and further purified on a MonoQ column. The concentrated AXH-d3 pool contained still some ß-galactosidase and $\alpha$-galactosidase activity. These could be removed by size-exclusion chromatography with a

Sephacryl S-200 column. The obtained AXH-d3 pool was found to be electrophoretically pure (not shown). The AXH-m23 pool was also applied onto a Sephacryl S-200 column and eluted as a highly pure fraction with only trace amounts of ß-galactosidase activity.

**Table 1.** Scheme of the purification of AXH-d3 and AXH-m23 from the Cell extract from *Bi. adolescentis*. U/g: Units per gram protein, U: Units

> Cell extract of *Bi. adolescentis*
> Resp. specific activity AXH-d3 and AXH-m23: 38 and 31 U/g
> Resp. total activity AXH-d3 and AXH-m23: 37528 and 30900 U

> Q-Sepharose HP 'High Load' XK 26/10, V=53 ml, flow rate: 8.33 ml/min
> Buffer A: 20 mM piperazine pH 6.5
> Buffer B: 20 mM piperazine pH 6.5+ 1M NaCl
> Resp. specific activity AXH-d3 and AXH-m23: 66 and 31 U/g
> Resp. total activity AXH-d3 and AXH-m23: 37528 and 30900 U

> Q-Sepharose HP 'High Load' XK 26/10, V=53 ml, flow rate: 8.33 ml/ min
> Buffer A: 20 mM piperazine pH 6
> Buffer B: 20 mM piperazine pH 6 + 1 M NaCl
> Resp. specific activity AXH-d3 and AXH-m23: 324 and 260 U/g
> Resp. total activity AXH-d3 and AXH-m23: 15660 and 12578 U

> BioGel Hydroxyapatite 26/10, V=53 ml, flow rate: 1.5 ml/min
> Buffer: 20 mM phosphate buffer pH 6
> Buffer B: 200 mM phosphate buffer pH 6
>
> 1. AXH-d3                                          2. AXH-m23
> Specific activity: 9400 U/g             Specific activity:  271 U/g
> Total activity: 710 U                       Total activity: 556 U

| | |
|---|---|
| Mono Q HR 5/5, V=1 ml, 0.5 ml/min<br>20 mM piperazine pH 6.5<br>20 mM piperazine pH 6.5 + 1 M NaCl<br>Specific activity AXH-m23: 11200 U/g<br>Total activity AXH-m23: 650 U | Mono Q HR 5/5, V=1 ml, 0.5 ml/min<br>20 mM piperazine pH 6.5<br>20 mM piperazine pH 6.5 + 1 M NaCl<br>Specific activity AXH-m23: 3455 U/g<br>Total activity AXH-m23: 104 U |
| Sephacryl S-200 HR16/60, V=319<br>20 mM phosphate buffer + 0.15 M NaCl<br>1ml/ min<br>Specific activity AXH-d3: 239000 U/g<br>Total activity AXH-d3: 430 U | Sephacryl S-200 HR16/60, V=319<br>20 mM phosphate buffer + 0.15 M NaCl<br>1 ml/min<br>Specific activity AXH-m23: 80000 U/g<br>Total activity AXH-m23: 20 U |

## Partial characterisation of AXH-d3 and AXH-m23

The molecular mass of AXH-d3 and AXH-m23 was respectively 100 kD and 160 kD, based on elution behaviour on the Sephacryl S-200 column. Both enzymes have an optimal activity at pH 6 using McIlvain Buffers. When piperazine buffer pH 6 was used, the optimal temperature for AXH-d3 was 30°C and for AXH-m23 37°C. The enzymes were active towards arabinoxylan-derived oligosaccharides, whereas AXH-d3 was also active against arabinoxylan polymer. They displayed no activity towards *p*-NP-ara*f*, sugar beet arabinan, soy arabinogalactan, and oligosaccharides derived from soy arabinogalactan and arabinan. AXH-m23 was also not active against arabinoxylan from wheat flour.

## Arabinose release from arabinoxylan polymers

The action of AXH-d3, AXH-m23, and AXH-m towards polymeric wheat arabinoxylan with an arabinose/xylose ratio of 0.4 was studied. AXH-m23, AXH-d3, AXH-m, and a combination of the latter two, released respectively 0, 25%, 40% and 90% of the arabinose present. AXH-m23 showed also no activity towards the arabinoxylan when pretreated with AXH-d3 or AXH-m.

Highly substituted (glucurono)-arabinoxylans, extracted from wheat bran (Schooneveld-Bergmans et al, 1999), were used to study the influence of the substitution with arabinose, xylose, and/or glucuronic acid on the enzymatic degradability. In Table 2 the release of arabinofuranosyl residues from the different fractions by AXH-d3 alone and in combination with AXH-m is given. AXH-d3 showed the highest activity against low-substituted arabinoxylan fractions. At higher substitution degrees, the AXH-d3 activity was found to decrease. Addition of AXH-m increased the release of arabinose in all fractions.

**Table 2.** Influence of substitution degree of (glucurono)arabinoxylan fractions from wheat bran on enzymatic degradability

| Fraction | A/X[a] | UA[b] | % release of total arabinose by AXH-d3 | % release of total arabinose by AXH-d3 and AXH-m |
|----------|--------|-------|----------------------------------------|--------------------------------------------------|
| BE   | 0.66 | 7.0 | 10 | 18 |
| BE30 | 0.20 | 1.1 | 33 | 43 |
| BE40 | 0.24 | 1.9 | 26 | 35 |
| BE50 | 0.35 | 1.7 | 28 | 35 |
| BE60 | 0.92 | 4.1 | 8  | 14 |
| BE70 | 1.02 | 3.9 | 5  | 11 |

a) A/X is the ratio arabinose/ xylose
UA: uronic acid
b) Expressed as percentage (mole per 100 mol), adapted from Schooneveld et al, 1999

## Mode of action towards wheat flour arabinoxylan

The different mode of action of AXH-d3 and AXH-m was studied using ^{1}H-NMR spectroscopy. The relevant parts of the ^{1}H-NMR spectra of untreated, AXH-d3 treated and (AXH-d3 + AXH-m) treated arabinoxylan polymer are shown in Fig 1. After incubation with AXH-d3 (Fig 1B), clear signals were observed at 5.283 and 5.397 ppm which showed that the arabinofuranosyl groups present were linked respectively to C-2 and C-3 of single-substituted xylopyranosyl residues (Viëtor et al, 1994). After (AXH-d3 + AXH-m) treatment no signals were detected (Fig 1C) which confirmed previous findings that this combination resulted in almost complete debranching.
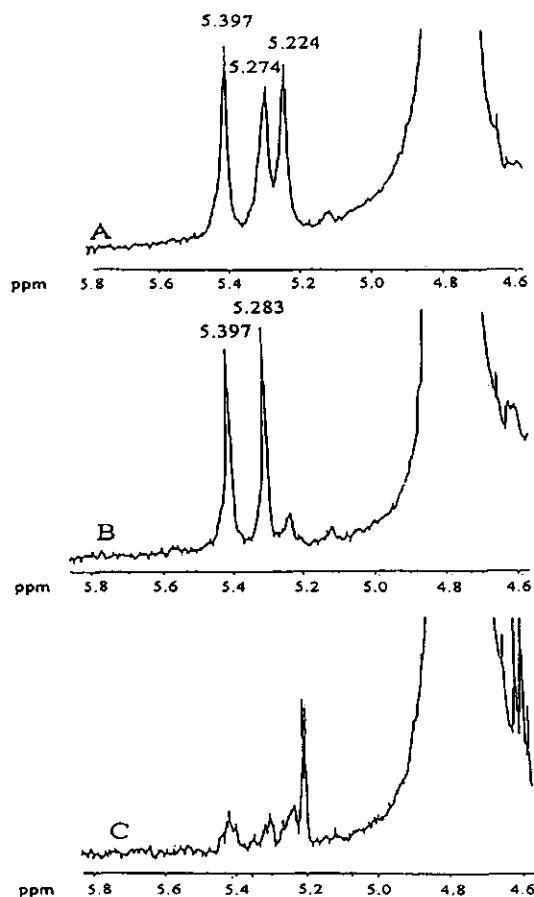


**Figure 1.** [1]H-Nuclear magnetic resonance (NMR) spectrum of untreated arabinoxylan polymer (A), arabinoxylan treated with AXH-d3 (B), and arabinoxylan treated with AXH-d3, AXH-m, and slightly with endo-xylanase I (C).

**Table 3.** Structures of arabinoxylan oligosaccharides and mode of action of AXH-d3, AXH-m and AXH-m23 on some arabinoxylan-derived oligosaccharides. Nomenclature according to Gruppen et al (1992) and Van Laere et al (1997b). $X_n$: $(\text{ß-D-Xyl}p1\rightarrow4\ \text{Xyl}p)_m$ with n=2-6, ◆: xylose, O: arabinose, * hypothesised structures obtained after digestion of AXH-d3 on the originating oligosaccharide

| oligomer structure (OS) | Enzymes → | Hypothetical OS | | Enzymes → | OS |
|---|---|---|---|---|---|
| | | 3.2 | ◆1→4◆ with 3↗1 O | AXH-m | $X_2$ |
| | | | | AXH-m23 | $X_2$ |
| | | 4.1 | ◆1→4◆1→4◆ with 3↗1 O | AXH-m | $X_3$ |
| | | | | AXH-m23 | $X_3$ |
| | | 5.2 | ◆1→4◆1→4◆ with 3↗3↗ 1 1 O O | AXH-m | $X_3$ |
| | | | | AXH-m23 | $X_3$ |
| | | 5.4 | ◆1→4◆1→4◆1→4◆ with 3↗1 O | AXH-m | $X_4$ |
| | | | | AXH-m23 | $X_4$ |
| | | 6.2 | ◆1→4◆1→4◆1→4◆ with 3↗3↗ 1 1 O O | AXH-m | $X_4$ |
| | | | | AXH-m23 | $X_4$ |
| 5.1 ◆1→4◆1→4◆ with 3 2 ↗↖ 1 1 O O | AXH-d3 | 5.1* | ◆1→4◆1→4◆ with 2↖ 1 O | AXH-m | $X_3$ |
| | | | | AXH-m23 | $X_3$ |
| 6.1 ◆1→4◆1→4◆1→4◆ with 3 2 ↗↖ 1 1 O O | AXH-d3 | 6.1* (5.5) | ◆1→4◆1→4◆1→4◆ with 2↖ 1 O | AXH-m | $X_4$ |
| | | | | AXH-m23 | $X_4$ |
| 6.3 ◆1→4◆1→4◆ with 3 2 3 ↗↖ ↗ 1 1 1 O O O | AXH-d3 | 6.3* | ◆1→4◆1→4◆ with 2 3 ↖ ↗ 1 1 O O | AXH-m | 6.3* |
| | | | | AXH-m23 | $X_3$ |
| 7.1 ◆1→4◆1→4◆1→4◆ with 3 3 2 ↗ ↗↖ 1 1 1 O O O | AXH-d3 | 7.1* | ◆1→4◆1→4◆1→4◆ with 3 2 ↗ ↖ 1 1 O O | AXH-m | $X_4$ |
| | | | | AXH-m23 | $X_4$ |
| 7.2 ◆1→4◆1→4◆1→4◆1→4◆ with 3 2 ↗↖ 1 1 O O | AXH-d3 | 7.2* | ◆1→4◆1→4◆1→4◆1→4◆ with 2↖ 1 O | AXH-m | $X_5$ |
| | | | | AXH-m23 | $X_5$ |
| 8.1 ◆1→4◆1→4◆1→4◆1→4◆ with 3 3 2 ↗ ↗↖ 1 1 1 O O O | AXH-d3 | 8.1* | ◆1→4◆1→4◆1→4◆1→4◆ with 3 2 ↗ ↖ 1 1 O O | AXH-m | $X_5$ |
| | | | | AXH-m23 | $X_5$ |
| 9.1 ◆1→4◆1→4◆1→4◆1→4◆ with 3 2 3 2 ↗↖ ↗↖ 1 1 1 1 O O O O | AXH-d3 | 9.1* | ◆1→4◆1→4◆1→4◆1→4◆ with 2 2 ↖ ↖ 1 1 O O | AXH-m | $X_5$ |
| | | | | AXH-m23 | $X_5$ |
| 10.1 ◆1→4◆1→4◆1→4◆1→4◆1→4◆ with 3 2 3 2 ↗↖ ↗↖ 1 1 1 1 O O O O | AXH-d3 | 10.1* | ◆1→4◆1→4◆1→4◆1→4◆1→4◆ with 2 2 ↖ ↖ 1 1 O O | AXH-m | $X_6$ |
| | | | | AXH-m23 | $X_6$ |

AXH-m and AXH-m23 were both active with the mixture of oligosaccharides obtained after incubation of the AXH-d3 treated polymer with endo-xylanase. However their mode of action differed (Table 3). In Figure 3 the elution profile of the reaction products obtained after incubation with both enzymes is given. Reaction products obtained after digestion with endo-xylanase of the AXH-m, AXH-d3 treated polymer were analysed with HPAEC and the elution patterns are shown in Fig 2. The oligomers formed from the AXH-d3 treated polymer were mainly single-substituted oligosaccharides (Fig 2 B, Table 3), while the oligomers formed on AXH-m treated polymer were mainly linear and double-substituted oligomers (Fig 2 C).
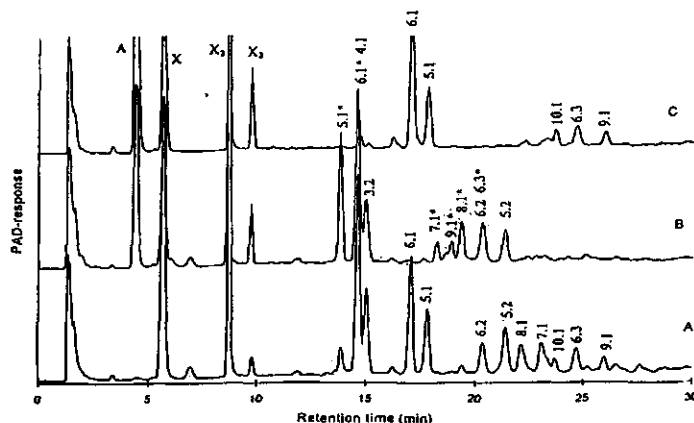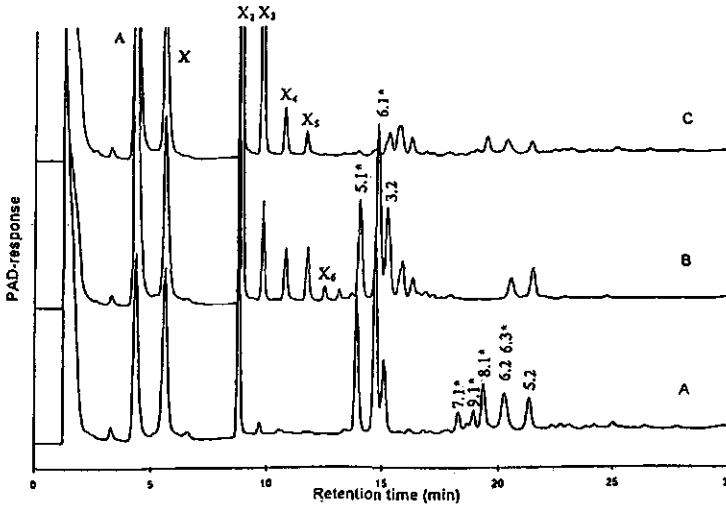


**Figure 2.** HPAEC analysis of the reaction products of arabinoxylan polymer incubated with endo-xylanase I (A), AXH-d3 and sequentially with endo-xylanase I(B), AXH-m and sequentially with endo-xylanase I (C). For explanation of peaks see Table 3. By degrading the purified oligosaccharides 5.1, 6.1, 7.1, 8.1, 9.1, and 6.3 by AXH-d3, peaks were observed at the same retention time as the * marked peaks.

## Mode of action towards differently substituted arabinoxylan oligomers

The activity of AXH-d3, AXH-m23, or AXH-m towards a mixture of oligosaccharides derived from untreated arabinoxylan and AXH-d3 treated arabinoxylan, and the purified oligosaccharide 6.1, 5.4, and 5.5 was investigated (Table 3). AXH-d3 showed no activity towards the oligosaccharides 5.4 and 5.5 but degraded the oligosaccharide 6.1 partially. AXH-m23 and AXH-m released using the same condition, approximately 3 times more arabinose from 5.5 as from 5.4. The two latter enzymes showed no activity against the oligosaccharide 6.1.

Different oligosaccharide mixtures representing a combination of double-substituted and single-substituted arabinoxylooligosaccharides were incubated with AXH-d3, AXH-m23 and AXH to investigate the influence on branching on their mode of action. AXH-d3, AXH-m and AXH-m23 released respectively 25%, 40% and 40% of the total amount of arabinose present in the mixture of oligosaccharides derived by incubation of untreated arabinoxylan with endo-xylanase. HPAEC-analysis showed clearly that AXH-d3 was not at all active towards the

oligosaccharides containing arabinofuranosyl residues linked to the C-3 position of single-substituted xylopyranosyl residues, AXH-d3 was able to remove arabinofuranosyl residues from all oligosaccharides present in the mixture containing double-substituted xylopyranosyl residues (Table 3).



**Figure 3** HPAEC analysis of the degradation products of arabinoxylan-derived oligosaccharides formed by AXH-d3 and sequentially endo-xylanase I treatment. Blank (A), incubated with AXH-m (B), incubated with AXH-m23 (C). For explanation of peaks see Table 3 By degrading the purified oligosaccharides 5.1, 6.1, 7.1, 8.1, 9.1, and 6.3 by AXH-d3, peaks were observed at the same retention time as the * marked peaks.

## DISCUSSION

This paper describes the isolation and characterisation of two novel arabinoxylan arabinofuranohydrolases from *Bi. adolescentis* (AXH-d3 and AXH-m23). The production of both enzymes was induced by growth of *Bi. adolescentis* on the arabinoxylan oligosaccharides 6.1, 5.1, 7.2, and, to a lower extent, by xylose, xylobiose, and xylotriose. No induction could be observed when *Bi. adolescentis* was grown on glucose, arabinose, and arabinoxylan polymer, although AXH-d3 was active towards this latter substrate. Arabinofuranosidase from *Bacteroides ovatus* (Whitehead and Hespell, 1990) has been reported to be induced by arabinose and oat spelt arabinoxylan. However, this enzyme was not solely specific for arabinoxylan as it was also active towards *p*-NP-ara*f*.

AXH-d3 and AXH-m23 showed similar elution behaviour using anion-exchange chromatography but they could be separated on an HTP column. Their molecular mass (AXH-d3, 100 kD; AXH-m23, 160 kD) was higher as has been reported for other AXHs (Beldman et al, 1997). Both enzymes had the highest activity at 30-40 °C and pH 6.0, which are conditions common to the large bowel.

AXH-d3 and AXH-m23 were only active towards arabinoxylans and therefore denoted as arabinoxylan arabinofuranohydrolases. AXH-d3 released all arabinofuranosyl residues, linked to the C-3 position of double-substituted xylose residues, present in polymeric an oligomeric arabinoxylans from wheat flour. AXH-m23 only released arabinofuranosyl groups from single-substituted xylose residues in oligomeric arabinoxylans. This enzyme showed no activity against arabinoxylan polymer, in contrast to AXH-m which specifically released arabinofuranosyl residues from the C-2 and C-3 position of single-substituted xylose residues in both arabinoxylan polymer and oligomers (Kormelink et al, 1993b). AXH-m23 and AXH-m, released under the same condition 3 times more arabinose from the oligosaccharide 5.5 as from the oligosaccharide 5.4. Their mode of action towards a mixture of branched oligosaccharides differed. AXH-m23 in combination with AXH-d3 could release all the arabinofuranosyl residues from arabinoxylooligosaccharides, while AXH-m was hindered in its action when the C-3 single-substitution is adjacent to the non-reducing side of a C-2 substituted xylose.

Arafur A from *A. Niger* (Rombouts et al, 1988) and AXH-m23 showed activity towards arabinoxylan oligosaccharides but not towards arabinoxylan polymer. In this study was also found that Arafur A, under the same condition, released 3 time more arabinofuranosyl groups from the C-2 position of single-substituted xylose residues compared to the C-3 position. In contrast to Arafur A, AXH-m23 showed no activity towards other arabinose containing substrates.

Wheat flour arabinoxylan polymer with an arabinose/ xylose ratio of 0.4 was almost completely debranched by a combination of AXH-m and AXH-d3. Kormelink et al (1993b) showed that Arafur B from *A. awamori* also had some activity towards arabinofuranosyl residues linked to the C-2 or C-3 position of double-substituted xylose residues. In our study arabinoxylan polymer was treated intensively with Arafur B but no complete removal of the arabinofuranosyl residues, attached to C-2 or C-3 position of double-substituted xylose residues was observed.

Only part of the arabinofuranosyl groups from highly substituted (glucurono)arabinoxylan could be released by combined treatment with AXH-m and AXH-d3. Both enzymes are probably inhibited in their action due to a high degree of substitution (i.e. xylose and glucuronic acid side groups and significant amounts of non-terminal arabinose) of the wheat bran (glucurono)arabinoxylan (Schooneveld-Bergmans et al, 1999 ). Verbruggen (1996) reported that AXH-m also was hindered in its action when a glucuronic acid was attached to a xylose residue adherent to a single-substituted xylose residue. To improve further degradation of (glucurono)-arabinoxylans, a combination of other debranching enzymes will be needed.

In conclusion, it can be said that two new arabinoxylan arabinofuranohydrolases (AXH-d3 and AXH-m23), which are both specific for arabinoxylans, could be isolated from *Bi. adolescentis*. The two enzymes had a different mode of action towards arabinoxylans than other arabinoxylan arabinofuranohydrolases reported before. AXH-m23 is the only enzyme

reported which specifically removes arabinofuranosyl groups from single-substituted xylose residues in arabinoxylan oligosaccharides only.

In this study it was noticed that *Bi. adolescentis* also produces a ß-xylosidase which degrades the formed xylooligosaccharides. The combination of the ß-xylosidase, AXH-d3 and AXH-m23 allows *Bi. adolescentis* to utilise arabinoxylooligosaccharides as growth substrate.

## ACKNOWLEDGEMENTS

## REFERENCES

Beldman G, Osuga D, Whitaker JR (1996) Some characteristics of beta-D-xylopyranosidases, alpha-L-arabinofuranosidases and an arabinoxylan alpha-L-arabinofuranohydrolase from wheat bran and germinated wheat. J Cereal Science 23: 169-180

Bergmans MEF, Beldman G, Gruppen H, Voragen AGJ (1996) Optimisation of the selective extraction of (glucurono)arabinoxylans from wheat bran: use of barium and calcium hydroxide solution at elevated temperatures. J Cereal Science 23: 235-245

Graaf LH de, Broek HC van de, Ooijen AJJ van, Visser J (1994) Regulation of the xylanase-encoding *xlnA* gene of *Aspergillus tubigensis*. Mol Microbiol 12: 479-490

Gruppen H, Hoffmann RA, Kormelink FJM, Voragen AGJ, Kamerling JP, Vliegenthart JFG (1992) Characterisation by ^{1}H-NMR spectroscopy of enzymically-derived oligosaccharides from alkali-extractable wheat flour arabinoxylan. Carbohydr Res 233: 45-64

Hoffmann RA, Leeflang BR, Barse MMJ de, Kamerling JP, Vliegenhart JFG (1991) Characterisation by ^{1}H-NMR spectroscopy of oligosaccharides, derived from arabinoxylans of white endosperm of wheat, that contain the elements →4[α-L-Araf-(1→3)]-ß-D-Xylp-(1→or→4)[α-L-Araf-(1→2)][α-L-Araf-(1→3)]-ß-D-Xylp-(1→). Carbohydr Res 221: 63-81

Kellett LE, Poole DM, Ferreira LMA, Durrant AJ, Hazlewood GP, Gilbert HJ (1990) Xylanase B and an arabinofuransoidase from *Pseudomonas fluoresens* subsp. *cellulosa* contain identical cellulose-binding domains and are encoded by adjacent genes. Biochem J: 272: 369-376

Kormelink FJM, Searle-Van Leeuwen MJF, Wood TM, Voragen AGJ (1991a) (1,4)-ß-D-arabinoxylan arabinofuranohydrolase: a novel enzyme in the bioconversion of arabinoxylan. Appl Microbiol Biotechnol 35: 231-232

Kormelink FJM, Searle-Van Leeuwen MJF, Wood TM, Voragen AGJ (1991b) Purification and characterization of an (1-4)-ß-D-arabinoxylan arabinofuranohydrolase from *Aspergillus awamori*. Appl Microbiol Biotechnol 35: 753-758

Kormelink FJM, Searle-Van Leeuwen MJF, Wood TM, Voragen AGJ (1993a) Purification and characterization of three endo-(1,4)-ß-xylanases and one ß-xylosidase from *Aspergillus awamori*. J Biotechnol 27: 249-265

Kormelink FJM, Gruppen H, Voragen AGJ (1993b) Mode of action of (1,4)-ß-D-arabinoxylan arabinofuranohydrolase (AXH-m) and α-L-arabinofuranosidases on alkali-extractable wheat-flour arabinoxylan. Carbohydr Res 249: 345-353

Rombouts FM, Voragen AGJ, Searle- Van Leeuwen MJF, Geraeds CCJM, Schols HA, Pilnik W (1988) The arabinanases of *Aspergillus niger*- Purification and characterization of two α-L-arabinofuranosidases and an endo-1,5-α-L-Arabinanase. Carbohydr Polym 9: 25-47

Schooneveld-Bergmans MEF, Beldman G, Voragen AGJ (1999) Structural features of glucuronoarabinoxylans extracted from wheat bran by barium hydroxide. J Cereal Science 29: 63-75

Sedmak JJ, Grossberg SE (1977) A rapid sensitive and versatile assay for protein using Commassie Brilliant Blue G250. Anal Biochem 79: 544-552

Van Laere KMJ, Bosveld M, Schols HA, Beldman G, Voragen AGJ (1997a) Fermentative degradation of plant cell wall derived oligosaccharides by intestinal bacteria. In: Hartemink R. (compiler) Non-digestible oligosaccharides: healthy food for the colon? Proceedings of a conference held in Wageningen, the Netherlands, 37-46

Van Laere KMJ, Beldman G, Voragen AGJ (1997b) A new arabinofuranohydrolase from *Bifidobacterium adolescentis* able to remove arabinofuranosyl residues form double-substituted xylose units in arabinoxylan. Appl Microbiol Biotechnol 47: 231-235 (Chapter 3)

Verbruggen MA (1996) Glucuronoarabinoxylans from sorghum grain. PhD thesis, Wageningen Agricultural University, The Netherlands

Viëtor RJ, Hoffmann RA, Angelino SAGF, Voragen AGJ, Kamerling JP, Vliegenthart JFG (1994) Structures of small oligomers liberated from barley arabinoxylans by endoxylanase from *Aspergillus awamori*. Carbohydr Res 254: 245-255

Whitehead TR, Hespell RB (1990) The genes for three xylan-degrading activities from *Bacteroides ovatus* are clustered in a 3.8-Kilobase region. J Bacteriol 172, 2408-2412

# 5

# Hydrolase and transgalactosylation activity of Bifidobacterium adolescentis α-galactosidase

*Bifidobacterium adolescentis*, a gram-positive saccharolytic bacterium found in the human colon, can, alongside other bacteria, utilise stachyose *in vitro* thanks to the production of an α-galactosidase. The enzyme was purified from the cell-free extract of *Bi. adolescentis* DSM 20083[T]. It was found to act with retention of configuration (α→α), releasing α-galactose from *p*-nitrophenyl galactoside. This hydrolysis probably operates with a double displacement mechanism, and is consistent with the observed glycosyl transferase activity.

As α-galactosides are interesting substrates for bifidobacteria, we focussed on the production of new types of α-galactosides using the transgalactosylation activity of *Bi. adolescentis* α-galactosidase. Starting from melibiose, raffinose and stachyose oligosaccharides could be formed. The transferase activity was highest at pH 7 and 40°C. Starting from 300 mM melibiose a maximum yield of 33% oligosaccharides was obtained. The oligosaccharides formed from melibiose were purified by size-exclusion chromatography and their structure was elucidated by NMR-spectroscopy in combination with enzymatic degradation and sugar linkage analysis. The trisaccharide α-D-Gal*p*- (1→6)- α-D-Gal*p*- (1→6)-D-Glc*p* and tetrasaccharide α-D-Gal*p*- (1→6)- α-D-Gal*p*-(1→6)- α-D-Gal*p*-(1→6)-D-Glc*p* were identified, and this indicates that the transgalactosylation to melibiose occurred selectively at the C-6 hydroxyl group of the galactosyl residue. The trisaccharide α-D-Gal*p*- (1→6)- α-D-Gal*p*- (1→6)-D-Glc*p* formed could be utilised by various intestinal bacteria, including various bifidobacteria, and might be an interesting pre- and synbiotic substrate.

## INTRODUCTION

Bifidobacteria are part of the resident microflora of the human colon. It is claimed that a high number of bifidobacteria is beneficial for the health of their hose. A high number of bifidobacteria may prevent colonisation of pathogens, and may have positive effects on intestine peristalsis, the immune system, cancer prevention, cholesterol metabolism and carbohydrate metabolism in the colon (Mitsuoka 1990; Ishibashi and Shimamura, 1993). To stimulate these bifidobacteria, specific carbohydrate sources, e.g. oligosaccharides, are used as bifidogenic factors). The soybean oligosaccharides (raffinose and stachyose) are reported to be bifidogenic substrates (Hayakawa et al. 1990). α-Galactosidase (EC 3.2.1.22) hydrolyses α-D-galactosidic bonds present in raffinose and stachyose. It has been reported that bifidobacteria possess high α-galactosidase activity (Chevalier et al. 1991; Scardovi 1986; Bezkorovainy and Miller-Catchpole 1989; Holdeman et al. 1977). Leder et al. (1999) purified the α-galactosidase from *Bifidobacterium adolescentis* and showed that the enzyme specifically hydrolyses α-galactosidic linkages, and α-(1→3) linkages were hydrolysed at a higher rate than α-(1→6) linkages.

By transglycosylation reactions using α-galactosidases, a new type of potential bifidogenic α-galactosides could be formed (Savel'ev et al. 1996; Hashimoto et al. 1995a,b). Although α-galactosidases are often described as hydrolases, some show also transferase activity. So far a lot of knowledge has become available for the production of ß-galactooligosaccharides using ß-galactosidases and lactose as a substrate (Zarate and Lopez-Leiva, 1990). Little research, however, has focused on the production of α-galactosides. The use of ß-galactosidase resulted in the formation of ß-galactooligosaccharides with different degrees of polymerisation and different linkage types (Fransen et al. 1999) and it may be possible to produce various α-galactooligosaccharides in a similar way. Van Laere et al. (1997b) have already observed that α-galactooligosaccharides could be formed from melibiose using *Bi. adolescentis* α-galactosidase.

As new α-galactosides might be interesting substrates for various intestinal bacteria, in particular those containing α-galactosidase activity, we are exploring the production of new types of potentially bifidogenic oligosaccharides by a transgalactosylation reaction using *Bi. adolescentis* α-galactosidase.

## MATERIALS AND METHODS

### Substrates

The *p*-nitrophenyl-glycosides (*p*-NP-glycosides) used for screening of glycosidase activities were obtained from Koch and Light, Ltd. (Haverhill, UK). Substrates for determining of α-galactosidase activity were raffinose (Fluka AG, Buhs SG, Switzerland), stachyose (Sigma

Chemicals Co., St. Louis, MI), melibiose (Jansens Chimica, Geel, Belgium), different galactomannans (Diagum GH, Diagum LBG and Taragum all from Diamalt GmbH, München, Germany), a galactomannan digest obtained by degradation with Gammanase, (NOVO Nordisk A/S, Bagsvaerd, Denmark) (an endo-mannanase containing enzyme preparation), α-D-Gal$p$-(1→3)-D-Gal$p$ and α-D-Gal$p$-(1→3)-ß-D-Gal$p$-(1→4)-D-Glc$p$, (Dextra Laboratories Ltd., Reading, United Kingdom). Globotriosylceramide and bovine thyroglobulin were obtained from Sigma Chemicals Co. (St. Louis, MI). Transgalactooligosaccharides were kindly provided by Borculo Whey Products (Borculo, The Netherlands).

## Strains

All intestinal strains were from the culture collection from our Food Science Group. Most strains were of human origin whereas some originated from porcine feces (Hartemink et al. 1996). *Bi. adolescentis* DSM 20083 was obtained from the Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH (DSMZ, Braunschweig, Germany).

## Fermentation of stachyose and 6'galactosyl melibiose

Various strains were screened for fermentation of stachyose and 6'galactosyl melibiose fermentation. The bacteria were precultured anaerobically in thioglycollate medium (Oxoid) containing 0.5% (w/v) glucose at 37°C for 24-48 h. Samples of 30µl of these cultures were added to 1 ml glucose free thioglycollate medium containing 0.5% (w/v) stachyose (Merck, Darmstadt, Germany) or 0.5% (w/v) 6'galactosyl melibiose. Thioglycollate medium and the sugar solutions were sterilised separately for 15 min at 121°C. The mixtures were incubated anaerobically for 48 h at 37°C. After incubation the pH was measured using a micro pH meter (Sentron, Roden, The Netherlands), the cultures were centrifuged and the supernatants were heated for 10 min at 100°C. The degradation of stachyose and 6'galactosyl melibiose after 48 h fermentation was monitored in the supernatants by High-Performance Anion-Exchange Chromatography (HPAEC).

## Purification of the α-galactosidase from Bi. adolescentis DSM 20083

### *Preparation of the crude cell extract*

*Bi. adolescentis* DSM 20083[T] was grown in 5000 ml M17 broth supplemented with 0.5 % (w/v) transgalactooligosaccharides. The enzyme-extract was prepared as described by Van Laere et al. (1997a).

### *Purification procedure of α-galactosidase*

α-Galactosidase was purified from the crude enzyme extract from *Bi. adolescentis*. The purification involved anion-exchange chromatography (Q-sepharose), adsorption-chromatography (BioGel HTP-hydroxyapatite column) and size-exclusion chromatography (Sephacryl S-200 column).

### *Enzyme assays*

α-Galactosidase activity was determined by the rate of hydrolysis of 0.02 % (w/v) p-nitrophenyl α-D-galactopyranoside (*p-NP-gal*) at 40°C and pH 6.5 (50 mM phosphate buffer). The increase in absorbance at 405 nm was measured spectrophotometrically. One unit of enzyme activity (U) was defined as 1 µmol of galactose liberated per minute.

The activity of α-galactosidase with various oligosaccharides, polysaccharides and glycoconjugate substrates was measured by HPAEC as a decrease in the relative surface area of the oligosaccharide peaks or, for the polysaccharides and glycoconjugates, as the formation of galactose.

Transferase activity was measured by HPAEC and size-exclusion chromatography after incubation of 100 µl 30-300 mM oligosaccharide (melibiose, raffinose or stachyose) with 0.2 U α-galactosidase at 40°C, pH 8 (McIlvain buffer) for different lengths of time.

## Characterisation of the α-galactosidase

### *pH and temperature optimum for hydrolase and transferase activity*

The optimum temperature for the α-galactosidase was determined by incubation of 3 mM raffinose or 300 mM raffinose solution in 50 mM phosphate buffer pH 6.0 at 20, 30, 35, 40, 45, 50, 60 °C for 1 h. The optimum pH for the α-galactosidase was determined by incubating 3 mM raffinose or 300 mM raffinose solution in citrate-phosphate buffers in the pH range of 3.0 up to 8.0 for 1 h at 40°C.

### [1]*H NMR analysis for establishing of the stereochemical course of hydrolysis*

Both the α-galactosidase (approx. 1.5 U) and *p*-NP-gal (5 mg) were freeze-dried twice from 99.9 % $D_2O$ and once from 99.96 % $D_2O$ (Cambridge Isotope Laboratories, Andover, Mass.) in order to exchange labile ^{1}H atoms for D. The substrate was dissolved in 0.7 ml 99.96 % $D_2O$ in a 5 mm NMR tube, and equilibrated at 30°C and the initial spectrum was recorded. Enzyme (50 µl in 99.96 % $D_2O$) was added and the stereochemical course of hydrolysis was followed by collection of ^{1}H NMR spectra during the incubation.

^{1}H NMR spectra were recorded at 30°C in a Brüker DPX-400 spectrometer operating at 400 MHz and equipped with a 5 mm ^{1}H/^{13}C probe. Spectra were referenced to the residual HDO

resonance at 4.74 ppm. Each spectrum was acquired with 56 transients and 2 dummy transients using 32796 data points over the 4000 Hz spectral width. The plotted spectra were normalised to the peak area of the HDO resonance.

### Isolation of the transfer products formed by transgalactosylation of melibiose with an α-galactosidase

300 mM (1 ml) melibiose was incubated with 0.4 U α-galactosidase at 40°C and in a 50 mM citrate-phosphate buffer pH 8 for 12 h. Boiling for 10 min stopped the reaction. The reaction mixture was separated on a BioGel P-2 column (100*2.8 cm) at 60°C using a Pharmacia Hiload system equipped with a P50 pump. A SHODEX RI-72 refractive index detector monitored the column effluent (0.5 ml/min). The collected fractions were analysed by HPAEC.

### NMR-spectroscopy of the oligosaccharides

One dimensional $^1$H NMR, one dimensional $^{13}$C NMR, two dimensional correlated spectroscopy (COSY), two dimensional total correlation spectroscopy (TOCSY), and two dimensional heteronuclear multiple bond correlation spectroscopy (HMBC) spectra were performed as described by van Casteren et al (1999) and Fransen et al (1998).

These experiments were carried out on a Brüker AMX-500 spectrometer located at the Wageningen NMR-centre. Chemical shifts are expressed in parts per million relative to internal acetone: $\delta$=2.225 ppm for $^1$H and $\delta$=31.077 ppm for $^{13}$C. For small sample amounts, Shigemi tubes (Campro Scientific, Veenendal, The Netherlands) with a sample volume of 190 µl were used.

## Analytical methods

### Gel electrophoresis

Electrophoresis was carried out with a PhastSystem (Pharmacia) according to the instructions of the supplier. The molecular mass was estimated by SDS-PAGE on a 10-15% polyacrylamide gel (Pharmacia). The isoelectric points were deduced from a pH 3 to 9 isoelectric focusing gel using the standards from the broad isoelectric point calibration kit (Pharmacia). The gels were stained with Coomassie brilliant blue R-250.

### HPAEC

HPAEC was performed using a Dionex Bio-LC system (Dionex, Sunnyvale, Ca) equipped with a Dionex CarboPac PA-1 (4*250 mm) and a Dionex pulsed electrochemical detection detector (PED) in the pulsed amperometric detection mode (PAD). The elution involved a gradient of sodium acetate in 100 mM NaOH as follows: 0 to 30 min, 0 to 200 mM; 30 to 35

min, 1000 mM; 35 to 50 min, 0 mM. For the separation of raffinose, stachyose, and verbascose the gradient was slightly adapted: 0 to 20 min, 0 to 60 mM.

## RESULTS

### Fermentation of stachyose

*In vitro* fermentative degradation of stachyose by various intestinal bacteria was studied. The results of the fermentation and degradation are summarised in Table 1. After 48h of fermentation the pH was measured. A final pH of 0.5 unit below the control (pH 6.3) was initially considered positive. Using this criteria 23 strains were found positive for fermentation of stachyose. Bifidobacteria reached the lowest pH after growth on stachyose compared to other bacteria tested. Whether this was related to stachyose fermentation was determined using HPAEC. Stachyose fermentation occurred only with *Bifidobacterium adolescentis, Bi. infantis, Bi. breve, Bi. pseudolongum, Bi. subtile, Bi. thermophilum, Bacteroides fragilis, B. ovatus, L. reuteri, L. acidophilus,* and *C tertium.* The degree of stachyose fermentation after 48h is also given in Table 1 and ranged from almost complete fermentation (*Bi. adolescentis*) to limited fermentation (*L. acidophilus*). *B. ovatus* and *B. fragilis* also fermented stachyose and different unknown degradation products accumulated.

As *Bi. adolescentis* was also one of the bacteria which dominated after culturing faecal inocula on stachyose, the α-galactosidase involved in the degradation of stachyose was purified and characterised.

### Purification and characterisation of α-galactosidase

*Bi. adolescentis* was grown on transgalactooligosaccharides until stationary growth and cells were harvested by centrifugation. After washing of the cells and sonication the enzyme extract (160 ml) contained 2 g of protein and had a total α-galactosidase activity of 2530 U with *p*-NP-gal.

The α-galactosidase was bound to a Q-Sepharose column, which had been equilibrated with 20 mM Bis-Tris buffer pH 7 and eluted at a NaCl concentration of 400 mM. The fractions having the highest activity were collected and further purified on a BioGel HTP hyroxyapatite column. The α-galactosidase eluted at a phosphate concentration of 75 mM. These fractions were pooled and concentrated on an anion exchange MonoQ HR 5/5 column. Further purification involved size-exclusion chromatography on a Sephacryl S-200 column. After these purification steps the α-galactosidase was obtained pure as judged from SDS-PAGE electrophoresis analysis (data not shown). The α-galactosidase was characterised for physico-chemical properties and substrate specificity. The enzyme had a specific activity of 23.2 U/mg and an optimal activity at pH 6 and 45°C.

**Table 1**: Ability of selected faecal bacteria to degrade and ferment stachyose

| Bacteria | pH | DF* | Bacteria | pH | DF* | bacteria | pH | DF* |
|---|---|---|---|---|---|---|---|---|
| *Bi. adolescentis* DSM 20083 | 4.4 | + 95 | *L.acidophilus* DSM 20079 | 6.2 | + 45 | *C. histolyticum* ATCC 19401 | 5.7 | - |
| *Bi. infantis* DSM 20223 | 4.2 | + 98 | *L.fermentum* | 6.3 | - | *C. butyricum* | 6.1 | - |
| *Bi. breve* DSM 20219 | 4.2 | + 85 | *L.reuteri* DSM 20016 | 5.4 | + 78 | *C. tertium* | 5.1 | + 97 |
| *Bi. suis* DSM 20211 | 5.6 | - | *B. thetaiotaomicron* DSM 2255 | 6.0 | - | *C. beijerinckii* | 6.3 | - |
| *Bi. bifidum* DSM 20082 | 6.1 | - | *B. ovatus* DSM 1896 | 5.0 | + 35 | *Ac. viscosus* | 5.0 | - |
| *Bi. bifidum* ATCC 35914 | 6.0 | - | *B. fragilis* DSM 2151 | 5.0 | + 97 | *Ac. naeslundii* | 6.2 | - |
| *Bi. animalis* DSM 20104 | 5.5 | - | *E. coli* | 5.7 | - | *Sa. bredeny* | 5.8 | - |
| *Bi. pseudocatenulatum* DSM 20438 | 6.0 | - | *Kl. pneumoniae* | 5.2 | - | *F. mortiferum* ATCC 9817 | 4.9 | + |
| *Bi.pseudolongum* subsp. | 3.9 | + 89 | *Klebsiella* spp | 5.0 | - | *Y. enterocolitica* | 5.5 | - |
| *pseudolongum*. DSM 20099 | | | *C. perfringens* | 6.2 | - | | | |
| *Bi. subtile* DSM 20096 | 4.3 | + 95 | *C. difficile* ATCC 19397 | 5.5 | - | | | |
| *Bi. thermophilum* DSM 20210 | 5.5 | + 30 | *Clostridium* spp. | 6.3 | - | | | |

DF: degree of fermentative degradation; + fermentation of stachyose and the percentage of stachyose which is fermented after 48h of incubation, - no degradation, pH; pH value after fermentation

Bi.: Bifidobacterium, B.: Bacteroides, C.: Clostridium, Kl.: Klebsiella, Sa.: Salmonella, Y: Yersinia, F.: Fusobacterium, Ac.: Actinomycetes, L.:Lactobacillus, E.: Escherichia

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The molecular mass, as estimated using Superose 12, was in the range of 330 kDa. SDS-PAGE revealed a single protein band at 83 kDa, suggesting that the enzyme had a tetrameric form. The α-galactosidase was tested on several substrates and was found to be active with the oligosaccharides melibiose, α-D-Gal*p*-(1→3)-D-Gal*p*, raffinose, α-D-Gal*p*-(1→3)-ß-D-Gal*p*-(1→4)-D-Glc*p*, stachyose and verbascose, but not towards galactomannan derived oligosaccharides. The α-galactosidase was also inactive with the terminal galactosyl units of the glycoconjugates bovine thyroglobulin and globotriaosylceramide and with galactomannan with different degrees of galactose substitution.

## Stereochemical course of hydrolysis

The stereochemical course of hydrolysis was measured by $^1$H NMR spectroscopy. Fig 1 shows the partial $^1$H NMR spectra of the anomeric region during α-galactosidase hydrolysis of *p*-NP-gal, illustrating the stereochemical course of the reaction.
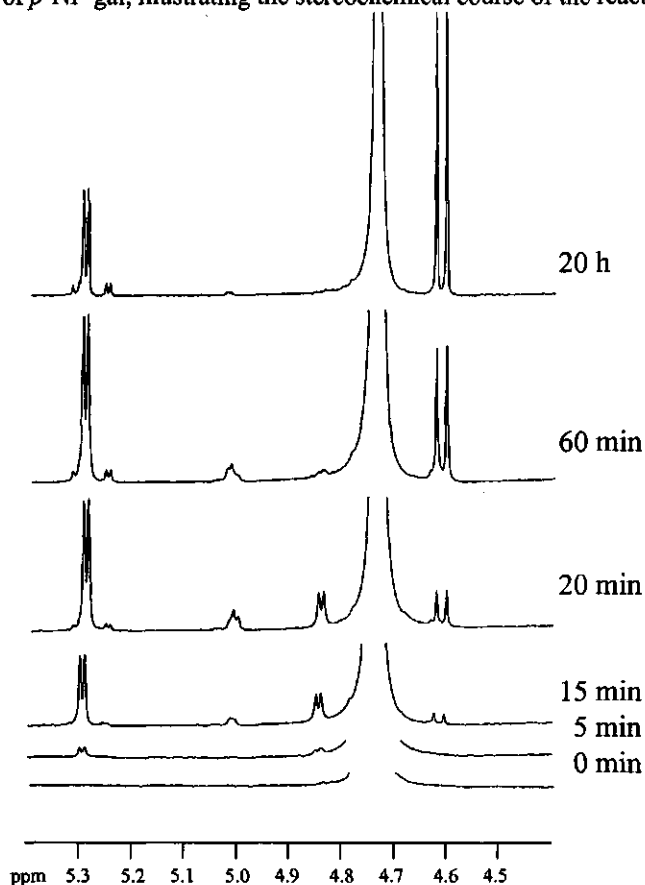


**Figure 1**. Partial $^1$H-NMR spectra showing the stereochemical course of hydrolysis of *p*-NP-gal by *Bi. adolescentis* α-galactosidase.

During the initial minutes of the incubation, a doublet at 5.30 ppm ($J$ =3.7 Hz) characteristic for H-1α of galactose (Izumi 1971) appeared and increased rapidly in intensity. A doublet at 4.62 ppm ($J$ =7.9 Hz), assigned to H-1ß (Izumi 1971) only became noticeable later in the incubation, and almost certainly arose from the mutarotation of the α-anomer initially formed. After 20 min the relative intensities of these α- and ß-anomer resonances were 4: 1, far from the expected mutarotational equilibrium ratio of approximately 1: 2.2 (Angyal and Pickles 1972) that was observed after 20 h. This clearly indicates that the enzyme hydrolysis occurs with retention of configuration (α→α).

Apart from the appearance of the α- and ß-galactopyranose anomeric resonances at 5.30 ppm and 4.62 ppm, some other resonances of interest occurred in the anomeric region during the hydrolysis of *p*-NP-gal. The most notables appeared at 5.26 ppm and 5.31 ppm and were caused by the presence of small amounts of α- and ß-galactofuranose respectively (Angyal and Pickles 1972) while resonances at 5.97 ppm (not shown) and 4.85 ppm possibly arose from transient products of glycosyl transferase reactions.

## Transferase activity of α-galactosidase

An optimum oligosaccharide synthesis was reached at pH 7 but was also rather high between pH 6 and pH 8 (75% of the optimal production at pH 7). At pH 6 and 7, some of the melibiose was hydrolysed while at pH 8 almost no galactose arose although 75% of the maximal oligosaccharide production was still reached. The temperature at which the highest amounts of oligosaccharides were formed was 40°C.

The conversion of melibiose into glucose, galactose and oligosaccharides with time as observed in a typical experiment starting from a 300 mM melibiose concentration, is given in Figure 2.
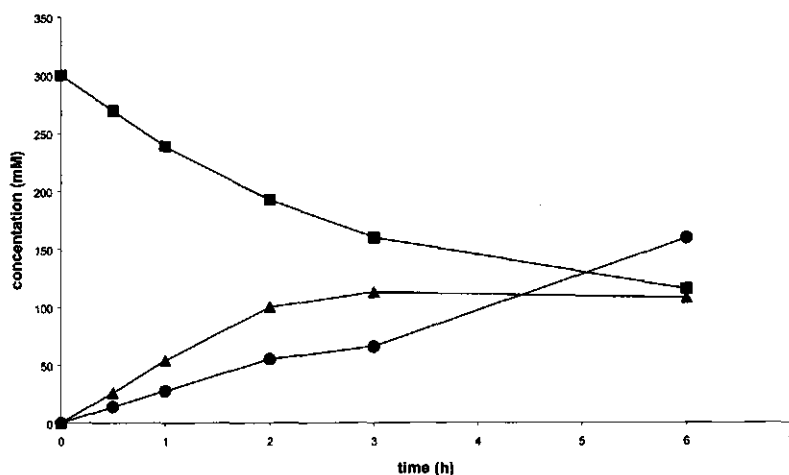


**Figure 2**. Time course of melibiose conversion and oligosaccharide synthesis starting from a 300 mM melibiose concentration. ■ melibiose, ▲ oligosaccharides, ● monosaccharide

Melibiose was hydrolysed to glucose and galactose and, in addition, oligosaccharides were produced. Following the transferase reaction over time it was observed that, after 3 h the highest oligosaccharide synthesis had occurred. A maximum yield of 33% oligosaccharides was formed under these conditions.

The transferase activity of α-galactosidase using different substrates was evaluated. Melibiose, raffinose, or stachyose was incubated separately for 6 h with 0.2 U enzyme. The transfer products were separated by HPAEC and size-exclusion chromatography. The α-galactosidase exhibited transgalactosylation activity with all three substrates tested. The different fractions obtained after size-exclusion chromatography showed that, from raffinose, mainly stachyose and, from stachyose, mainly verbascose was formed on the basis of retention behaviour on HPAEC. From melibiose an unknown tri- and tetrasaccharide were formed during the transferase reaction (Figure 3).
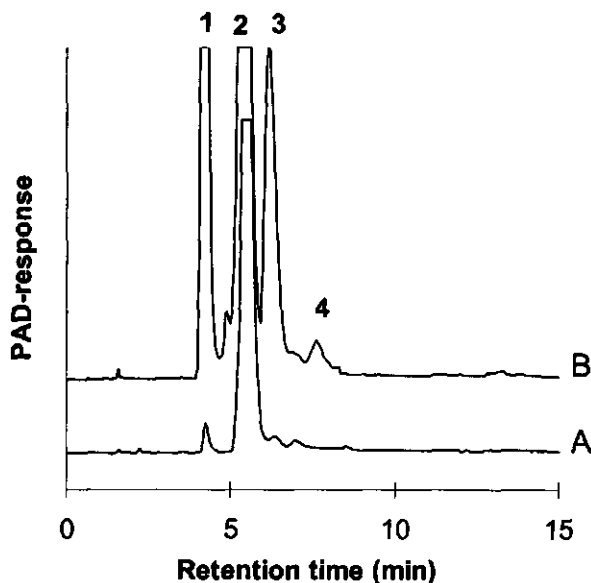


**Figure 3.** High-Performance Anion-Exchange chromatography elution pattern of products formed after transgalactosylation of melibiose with the α-galactosidase of *Bi. adolescentis*. 1: monosaccharides; 2: melibiose; 3: trisaccharide α-D-Gal*p*- (1→6)- α-D-Gal*p*- (1→6)-D-Glc*p*, PAD: pulsed amperiometric detection

These unknown products were isolated on a BioGel P-2 column after incubation on larger scale has been performed. The reaction mixture consisted of 18% monosaccharides, 61 % melibiose, 18 % trisaccharide and 3 % tetrasaccharide. The purity of the different fractions was checked by HPAEC. The different fractions (degree of polymerisation = 2-4) only consisted of one oligosaccharide of unknown structure. The structures of the trisaccharide and

tetrasaccharide were elucidated by NMR-spectroscopy in combination with sugar analysis, methylation analysis and enzymatic degradation studies.

NMR-spectroscopy of **trimer (1)**. The structure of the oligosaccharides was elucidated by combining information obtained from sugar linkage analysis, enzymatic degradation and NMR-spectroscopy. Methylation analysis demonstrated the presence of terminal Gal*p*, 1→6-linked Gal*p* and 6-substituted Glc*p* (not shown). Upon degradation of the trisaccharide, using the *Bi. adolescentis* α-galactosidase, melibiose and galactose were formed.

The 1D $^1$H -NMR spectrum of the **trimer (1)** (not shown) contained 3 resonances in the anomeric region, which were designated **A** H-1 -**B** H-1 - $\mathbf{C}^{\alpha/\beta}$ H-1. According to their intensities, the narrow doublet at δ 5.23 ppm ($\mathbf{C}^\alpha$ H-1, $^3J_{1,2}$ =3.8 Hz, 0.3 H) and the broader doublet at 4.66 ppm ($\mathbf{C}^\beta$ H-1, $^3J_{1,2}$ =8.1 Hz, 0.7 H) were assigned to the sugar residue at the reducing end. The third resonance (**A** H-1 + **B** H-1, $^3J_{1,2}$ =3.2-3.8 Hz, 2 H) consisted of a multiplet centred at δ 4.98 ppm and was assigned to two overlapping α-glycosyl residues. The 2D COSY and TOCSY measurements (not shown) allowed the assignment of the protons as is shown in Table 2. Since the TOCSY $\mathbf{C}^\beta$ H-1 track showed correlations with H-2, 3, 4, 5, 6a, 6b, residue **C** was identified as a glucose residue. The TOCSY H-1 tracks of residues **A** and **B** showed only cross-peaks to H-2, 3, 4, which, combined with the characteristic resonance positions of both H-4, allowed these to be identified as galactosyl residues.

**Table 2**: $^1$H NMR chemical shifts of the trimer, as determined from COSY and TOCSY, relative to the chemical shift of acetone at δ 2.225 ppm; z between 5.00 ppm and 4.96 ppm.

| Residue (1) | H-1 | H-2 | H-3 | H-4 | H-5 | H-6a | H-6b |
|---|---|---|---|---|---|---|---|
| A | z | 3.80 | 3.84 | 3.96 | 3.99 | 3.73 | 3.73 |
| B | z | 3.81 | 3.89 | 4.02 | 4.16 | 3.85 | 3.70 |
| $C^\alpha$ | 5.23 | 3.52 | 3.70 | 3.48 | 4.00 | 4.00 | 3.68 |
| $C^\beta$ | 4.66 | 3.23 | 3.46 | 3.49 | 3.63 | 3.95 | 3.73 |

    A         B         C

α-D-Gal*p*- (1→6)- α-D-Gal*p*- (1→6)-α/β-D-Glc*p*

The 1D $^{13}$C NMR spectrum of **trimer (1)** (not shown) showed 6 anomeric signals. On the basis of their chemical shifts, the C-1 signals at δ 93.0 ppm and 96.9 ppm were assigned to the glucosyl residue at the reducing end ($\mathbf{C}^\alpha$ and $\mathbf{C}^\beta$ respectively). Four closely spaced $^{13}$C resonances at δ 99, being in fact two pairs of α-galactopyranosyl $^{13}$C -1 resonances that reflect the anomeric equilibrium of the trimer belonged to the residues **A** and **B**.

The 2D $^{13}$C-$^1$H HMBC spectrum of the **trimer (1)** (Figure 4) recorded with a delay time of 80 msec allowed a nearly complete assignment of the $^{13}$C signals as summarised in Table 3.

**Table 3:** $^{13}$C NMR chemical shifts relative to the chemical shift of acetone C-1 ($\delta$ 31.077 ppm) (of the trimer as determined from the $^{13}$C- $^1$H HMBC spectrum, B(C$\alpha$): residue B when the C-residue is in the $\alpha$-position

| Residue (1) | C1 | C2 | C3 | C4 | C5 | C6 |
|---|---|---|---|---|---|---|
| A (C$\alpha$) | 98.86 | 69.08 | 70.29 | 70.05 | 71.79 | 61.96 |
| A (Cß) | 98.81 | 69.08 | 70.29 | 70.05 | 71.79 | 61.96 |
| B (C$\alpha$) | 98.89 | 69.26 | 70.22 | 70.17 | 69.62 | 67.37 |
| B (Cß) | 98.83 | 69.22 | 70.22 | 70.15 | 69.56 | 67.27 |
| C ($\alpha$) | 93.00 | 72.23 | 73.82 | 70.50 | 70.73 | 66.63 |
| C (ß) | 96.89 | 74.86 | 76.77 | 70.34 | 74.99 | 66.52 |

The C-6 resonances of residue **A** were found at $\delta$ 62.0 ppm. This position favours of the absence of a linkage at C-6 (Bock and Thøgersen 1982). The resonances of **B** C-6 and **C** C-6 were found between $\delta$ 66 ppm and 68 ppm indicating C-6 linkages to other residues. Therefore, residue **A** was assigned to the sugar residue at the non-reducing end.
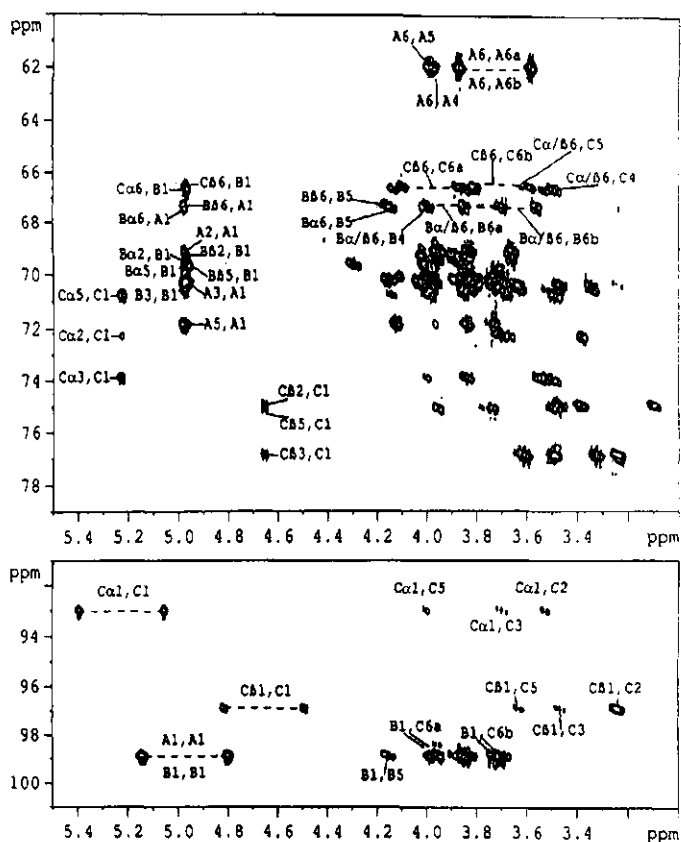


**Figure 4:** Partial 500 MHz 2 D $^{13}$C- $^1$H HMBC spectrum of **trimer (1)** in D2O at 27°C. The code Cß6, B1 corresponds to the coupling C C$^\beta$-6 and B H-1.

From this 2D $^{13}$C-$^{1}$H HMBC spectrum it can be inferred that **A** C-6 has only <u>intra</u>-residual correlations with **A** H-6a (A6, A6a (1J)), **A** H-6b (A6, A6b (1J)), **A** H-5 (A6, A5 (2J)) and **A** H-4 (A6, A4 (3J)) but not with **A** H-3 (4J), **A** H-2 (5J), and importantly, *not* with **A** H-1 (4J). The latter observation implicates that the strong cross-peaks between **B** $C^{\alpha/\beta}$-6 and one of the α-galactosyl H-1 protons (Bα6, A1 and Bβ6, A1) reflect correlation over the glycosidic bond between **B** $C^{\alpha/\beta}$ -6 and **A** H-1. The other correlations which were observed for **B** $C^{\alpha/\beta}$ -6 were intra-residual: with **B** H-6a (Bα/β6, B6a (1J)), **B** H-6b (Bα/β6, B6b (1J)), **B** H-5 (Bα/β6, B5 (2J)) and **B** H-4 ( Bα/β6, B4 (3J)), just as was observed for **A** C-6. The strong cross-peaks between $C^{\alpha/\beta}$ C-6 and one of the α-galactosyl H-1 protons (Cα/β6, B1) reflect the long range correlation across the other glycosidic bond in the trimer between **C** $C^{\alpha/\beta}$ -6 and **B** H-1. The other correlations observed for $C^{\alpha/\beta}$ C-6 were intra-residual: with **C** H-6a (Cα/β 6, C6a (1J)), **C** H-6b (Cα/β6, C6b (1J)), **C** H-5 (Cα/β 6, C5 (2J)) and **C** H-4 (Cα/β 6, C 4 (3J)).

The correlations of $C^{\alpha,\beta}$ C-1 were also nicely exposed and were only intra-residual: with **C** H-1 (Cα/β1, C1 (1J)), **C** H-2 (Cα/β 1, C2 (2J)), **C** H-3 (Cα/β 1, C3 (3J)) and **C** H-5 (Cα/β 1, C5 (3J)).

The associated reverse correlations over the glycosidic bonds between **A** C-1 and **B** H-6a and **B** H-6b over the glycosidic bond between **A** and **B**, and between **B** C-1 and **C** H-6a and **C** H-6b over the glycosidic bond between **B** and **C** can be distinguished in this 2D $^{13}$C-$^{1}$H HMBC spectrum but with cross-peaks intensities being comparable to the others. When the delay time was increased from 80 ms to 200 ms, all four correlations become prominent on the **A** C-1 and **B** C-1 track in the 2 D $^{13}$C- $^{1}$H HMBC spectrum, their intensities now exceeding those of the others.

The 2D COSY spectrum of stachyose was also recorded, stachyose being a tetramer composed of the **trimer (1)** and a fructose moiety linked to its reducing end. The trimer pattern was easily recognised in this spectrum, the glucose residue having now the α-pyranosyl configuration.

**NMR-spectroscopy of tetramer (2).** Because only low amounts of **tetramer (2)** were available no methylation analysis was performed. However it was observed that, upon degradation of **tetramer (2)** by *Bi. adolescentis* α-galactosidase, the **trimer (1)** and gal*p* were formed. The 1D $^{1}$H -NMR spectrum of the **tetramer (2)** again showed 3 resonances in the anomeric region, which were labelled A' H-1, A H-1, B H-1 and $C^{\alpha\beta}$ H-1. The anomeric region resembles that of the **trimer (1)** except for the shape of the resonance centred at δ 4.98 ppm, which was now composed of six narrow doublets due to the presence of a third α-galactosyl residue being 1→ linked to the trimer. From the 2D COSY spectrum (not shown) the $^{1}$H resonances of all protons could easily be assigned; they are summarised in Table 4.

**Table 4:** $^1$H NMR chemical shifts of the tetramer as determined from COSY, relative to the chemical shift of acetone at δ 2.225 ppm; z between 5.00 ppm and 4.96 ppm

| Residue (2) | H-1 | H-2 | H-3 | H-4 | H-5 | H-6a | H-6b |
|-------------|-----|-----|-----|-----|-----|------|------|
| A' | z | 3.81 | 3.85 | 3.98 | 3.99 | 3.74 | 3.74 |
| A | z | 3.81 | 3.86 | 4.02 | 4.19 | 3.85 | 3.71 |
| B | z | 3.82 | 3.90 | 4.01 | 4.15 | 3.88 | 3.66 |
| C$^\alpha$ | 5.23 | 3.52 | 3.70 | 3.49 | 4.01 | 4.01 | 3.68 |
| C$^\beta$ | 4.66 | 3.24 | 3.47 | 3.50 | 3.64 | 3.96 | 3.73 |

A'          A              B                 C

α-D-Gal*p*- (1→6)- α-D-Gal*p*- (1→6)- α-D-Gal*p*- (1→6)-α/ß-D-Glc*p*
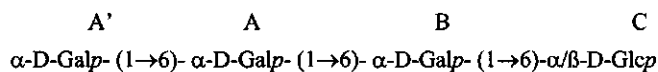
Inspection of Table 4 shows that insertion of one α-galactosyl residue of type **B** in the **trimer (1)** resulted in the formation of the **tetramer (2)**. Unfortunately, interresidual correlations could not be determined for a 2D $^{13}$C-$^1$H HMBC spectrum because of a too low signal/ noise ratio as result of the low sample concentration.

## Fermentation of α-D-Gal*p*- (1→6)- α-D-Gal*p*- (1→6)-D-Glc*p*

A selection of bacteria was tested for their ability to ferment 6'galactosyl melibiose (Table 5). The bifidobacteria tested were able to ferment the structure completely within 48h and a low pH was reached. Also all lactobacilli tested were able to ferment 6'galactosyl-melibiose, but not completely within the fermentation time.

**Table 5**: Fermentation of 6'galactosyl melibiose by bifidobacteria and *Lactobacilli* spp.

| Bacteria | PH | DF |
|----------|-----|------|
| *L.fermentum* | 5.5 | + 85 |
| *L.acidophilus* | 5.6 | + 85 |
| *L.casei* | 6.2 | + 77 |
| *Bi. breve* | 4.6 | + 98 |
| *Bi. longum* | 4.7 | + 98 |
| *Bi. infantis* | 4.6 | + 96 |
| *Bi. adolescentis* | 4.7 | + 96 |

DF: degree of fermentative degradation; + fermentation of 6'galactosyl melibiose and the percentage of 6'galactosyl melibiose which is fermented after 48h of incubation, - no degradation

## DISCUSSION

Raffinose and stachyose are regarded as prebiotic substrates, meaning that they can selectively stimulate the growth and/ or activity of a limited number of bacteria in the colon (Gibson and Roberfroid 1995). The high α-galactosidase levels of several intestinal bacteria, mainly bifidobacteria stimulated us to look for new prebiotic substrates that are not digested by intestinal enzymes and might be fermented by several intestinal bacteria. Raffinose and stachyose are probably the best-known α-galactosides and the fermentation of raffinose by different bacteria has been principally was studied (Benno et al. 1987; Sakai et al. 1987; Holdeman et al. 1977). We showed that several bifidobacteria, *L. reuteri, L. acidophilus, B. fragilis, B. ovatus, C tertium,* and *F. mortiferum* fermented stachyose, but the degree of fermentation and the degradation products formed differed. Several α-galactosidases have been purified from intestinal bacteria. The presence of a α-galactosidase was reported for some bifidobacteria (Garro et al. 1994; Roy et al. 1991; Desjardins et al. 1990, Leder et al. 1999), lactobacilli (Kandler and Weiss 1986; Mital 1973), some Bacteroides species (Valentine et al. 1991; Gherardini et al. 1985) and for *Clostridium perfringens* (Durance and Skura 1985) which are all important species in the large bowel.

*In vitro* fermentation experiments carried out using faecal inocula showed that bifidobacteria were one of the strains that dominated after faecal inocula were cultured on stachyose. Next to the bifidobacteria, *Bacteroides ovatus* also accumulated (data not shown). The α-galactosidase from *B. ovatus* was characterised by Gherardini et al. (1985) and recently the α-galactosidase *from Bi. adolescentis* was partially characterised by Leder et al. (1999). The α-galactosidase purified to homogeneity in this study by anion-exchange-, adsorption, and size-exclusion chromatography was found to have a molecular mass of 83 kDa by SDS-PAGE. On the other hand, the value obtained by size-exclusion chromatography was 330 kDa. The enzyme was optimally active at pH 6 and at 45°C. These results are comparable to those found by Leder et al (1999) and values found for the α-galactosidase from *Bi. infantis* (Roy et al. 1992) and *Bi. longum* (Garro et al. 1994). Since we used different assay conditions than Leder et al. (1999), the measured specific activity can not be compared with their data. Leder et al (1999) showed that the *Bi. adolescentis* α-galactosidase hydrolysed α-(1→3)-linkages at higher rates than α-(1→6)-linkages. In addition we showed that the enzyme was not active with galactomannan-derived oligosaccharides, polymeric galactomannan of various degrees of galactosyl substitution or α-galactoside linkages present in the glycoconjugates tested.

As α-galactosides are interesting substrates for bifidobacteria, we further focused on the production of new types of α-galactosides using *Bi. adolescentis* α-galactosidase in analogy to various ß-galactosides that have been prepared using the transgalactosylation activity of ß-galactosidases (Zarate and Lopez-Leiva 1990). Dumortier et al. (1994) showed that a ß-galactosidase from *Bi. bifidum* showed transferase activity. To the best of our knowledge there are no studies describing the synthesis of α-galactooligosaccharides using bacterial α-galactosidase. Transgalactosylation by α-galactosidase will probably not occur *in vivo* as the

substrate concentrations will be to low. α-Galactosidase may, however, be used for the production of α-galactosides, which might be used as a prebiotic growth substrate for intestinal bacteria.

*Bi. adolescentis* α-galactosidase was found to act with retention of configuration (α→α), releasing α-galactose from *p*-NP-gal. This hydrolysis probably operates through a double displacement mechanism (Sinnott 1990) and is consistent with the observed glycosyl transferase activity. A search of the literature found few similar stereochemical studies with other α-galactosidases, although the results obtained here are consistent with those found for an α-galactosidase isolated from coffee beans (Weiser et al. 1992).

A series of galactosyl oligosaccharides have been synthesised from melibiose by the α-galactosidase from *Bi. adolescentis*. NMR analysis of the oligosaccharides demonstrated that, from this action, the trisaccharide α-D-Gal*p*- (1→6)- α-D-Gal*p*- (1→6)-D-Glc*p* and the tetrasaccharide having the proposed structure α-D-Gal*p*- (1→6)- α-D-Gal*p*-(1→6)- α-D-Gal*p*-(1→6)-D-Glc*p* were obtained, indicating that the transgalactosylation to melibiose was found to occur selectively at the C-6 hydroxyl group of the galactosyl residue and that no oligosaccharides containing α-(1→3) or other linkages were formed. This is remarkable in view of the observed hydrolytic activity where it was shown that the enzyme hydrolysed α-(1→3)-linkages to a higher extent than α-(1→6) linkages (Leder et al., 1999). α-D-Gal*p*- (1→6)- α-D-Gal*p*- (1→6) -D-Glc*p* was also formed from melibiose by the α-galactosidase from *Trichoderma reesei* (Savel'ev et al. 1996).

In the presence of raffinose, α-galactosidase from *Bi. adolescentis* formed stachyose and, from stachyose, verbascose was obtained. Formation of stachyose as a transfer product from raffinose was also reported for α-galactosidases from *Streptococcus bovis*. There have been few reports of α-galactosidases that form α-(1→3) and α-(1→4)-linkages by transgalactosylation. The *Pycnoporus cinnaborinus* (Mitsutomi and Ohtakara 1988) α-galactosidase produced not only oligosaccharides of the raffinose family but also oligosaccharides containing α-(1→3)-galactosidic linkages, while oligosaccharides containing α-(1→4)-galactosidic linkages were produced by the transgalactosylation reaction of *Sachys affinis* α-galactosidase (Kato et al. 1982).

The trisaccharide α-D-Gal*p*- (1→6)- α-D-Gal*p*- (1→6)-D-Glc*p* formed could be fermented by various lactobacilli including *L. fermentum*, *L. acidophilus*, *L. casei* and bifidobacteria including *Bi. breve, Bi. longum, Bi. infantis* and *Bi. adolescentis*. α-D-Gal*p*- (1→6)- α-D-Gal*p*- (1→6)-D-Glc*p* can therefore be used as a synbiotic product, supporting the growth of exogenous added probiotic bacteria. However, whether this type of oligosaccharides can be used as a prebiotic substrate, thereby stimulating the growth of endogenous bifidobacteria and lactobacilli, needs further investigations.

## REFERENCES

Angyal SJ, Pickles VA (1972) Equilibria between pyranoses and furanoses. II. Aldoses. Austr J Chem 25: 1695-1710

Benno Y, Endo K, Shiragami N, Sayama K, Mitsuoka T (1987) Effect of raffinose intake on human fecal microflora. Bifidobacteria Microflora 6:59-63

Bezkorovainy A, Miller-Catchpole R (1989) Biochemistry and physiology of bifidobactera. CRC, Boca Raton, Fla

Bock K, Thøgersen H (1982) Nuclear magnetic resonance spectroscopy in this study of mono-and oligosaccharides. Ann Rep NMR Spectrosc 13: 1-57

Casteren WHM van, Kabel MA, Dijkema C, Schols HA, Beldman G, Voragen AGJ (1999) Endoglucanase V and a phosphatase from *Trichoderma viride* are able to act on modified exopolysaccharide from *Lactococcus lactis* subsp. *cremoris* B-40. Carbohydr Res (in press)

Chevalier P, Roy D, Savoie L (1991) X-alpha-Gal-based medium for simultaneous enumeration of bifidobacteria and lactic acid bacteria in milk. J Microbiol Methods 13:75-83

Desjardins ML, Roy D (1990) Growth of Bifidobacteria and their enzyme profiles. J Dairy Sc 73:299-307

Dumortier V, Brassart C, Bouquelet S (1994) Purification and properties of a beta-D-galactosidase from *Bifidobacterium bifidum* exhibiting a transgalactosylation reaction. Biotechnol Appl Biochem 19: 341-354

Durance T, Skura B (1985) Purification and characterization of a *Clostridium perfringens* alpha-galactosidase. J Food Science 50:518-522

Fransen CTM, Van Laere KMJ, Wijk AAC van, Brüll LP B, Dignum M, Thomas-Oates JET, Haverkamp J, Schols HA, Voragen AGJ, Kamerling JP, Vliegenthart JFG (1998). $α$-D-Glc$p$-(1↔1)-ß-D-Gal$p$-containing oligosaccharides, a new class of oligosaccharides produced by ß-galactosidase from lactose. Carbohydr Res 314:101-114

Garro MS, Giori GS de, Valdez GF de, Oliver G (1994) alpha-D-Galactosidase (EC 3.2.1.22) from *Bifidobacterium longum*. Letters in Appl Microbiol 19: 16-19

Gherardini F, Babcock M, Salyers AA (1985) Purification and characterisation of two alpha-galactosidases associated with catabolism of guar gum and other alpha-galactosides by *Bacteroides ovatus*. J Bacteriol. 161: 500-506

Gibson GR, Roberfroid MB (1995) Dietary modulation of the human colonic microbiota- introducing the concept of prebiotics. J Nutr 125: 1401-1412

Hartemink R, Van Laere KMJ, Mertens AKC, Rombouts FM (1996) Fermentation of xyloglucan by intestinal bacteria. Anaerobe. 2:223-230

Hashimoto H, Katayama C, Goto M, Okinaga T, Kitahata S (1995a) Enzymatic synthesis of $α$-linked galactooligosaccharides using the reverse reaction of a cell-bound $α$-galactosidase from *Candida guilliermonii* H-404. Biosci Biotechnol Biochem 59: 179-183

Hashimoto H, Katayama C, Goto M, Okinaga T, Kitahata S (1995b) Transgalactsylation catalyzed by bound $α$-galactosidase from *Candida guilliermonii* H-404. Biosci Biotech Biochem 59: 619-623

Hayakawa K, Mizutani J, Wada K, Masai T, Yoshihara I, Mitsuoka T (1990) Effect of soybean oligosaccharides on human fecal flora. Microbiol Ecol Health Dis 3: 293-303

Holdeman LV, Cato EP, Moore WEC (1977) Anaerobic laboratory manual, 4th edn. VPI Anaerobe laboratory, Blacksurg, V

Ishibashi N, Shimamura S (1993) Bifidobacteria: research and development in Japan. Food Technol 47: 126-136

Izumi K (1971) NMR spectra of some monosaccharides of galactopyranose series in deuterium oxide. Agric Biolog Chem 35:1816-1818

Kandler O, Weiss N (1986) In: Holt JG, Williams , Wilkins, (eds) Bergey's manual of systematic bacteriology. Vol 2. Baltimore, Md.

Kato K, Ikami T, Kono H, Yamauchi R, Ueno Y (1982) Transferase action of α-galactosidase from Tubers from *Stachys affinis*. Agric Biol Chem 46:1089-1090

Leder S, Hartmeier W, Marx SP (1999) α-galactosidase of *Bifidobacterium adolescentis* DSM 20083. Curr Microbiol 38:101-106

Mital BK, Shallenberger RS, Steinkraus KH (1973) α-Galactosidase activity of lactobacilli. Appl Microbiol 26: 783

Mitsuoka T (1990) Bifidobacteria and their role in human health. J Ind Microbiol 6: 263-268

Mitsutomi M, Ohtakara A (1988) Isolaton and identification of oligosaccharides produced from raffinose by transgalactosylation reaction of thermostable α-galactosidase from *Pycnoporus cinnabarinus* Agric Biol Chem 52: 2305-2311

Roy D, Chevalier P, Ward P, Savoie L (1991) Sugars fermented by *Bifidobacterium infantis* ATCC 27920 in relation to growth and alpha-galactosidase activity. Appl Microbiol and Biotechnol 34: 653-655

Roy D, Blanchette L, Savoie L, Ward P, Chevalier P (1992) alpha- and beta-galactosidase properties of *Bifidobacterium infantis*. Milchwissenschaft 47:18-21

Sakai K, Tachiki T, Kumagai H, Tochikura T (1987) Hydrolysis of α-galactosyl oligosaccharides in soymilk by α-galactosidase of *Bifidobacterium breve* 203. Agric Biol Chem 51: 315

Savel'ev AN, Ibatyllin FM, Eneyskaya EV, Kachurin AM, Neustroev KN ( 1996) Enzymatic properties of α-galactosidase from *Trichoderma reesei*. Carbohydr Res 296: 261-273

Scardovi V (1986) Genus Bifidobacterium. In: Sneath P, et al (eds) Bergey's manual of systematic bacteriology. Vol 2 Williams and Wilkins. Baltimore, Md.

Sinnott ML (1990). Catalytic mechanisms of enzymic glycosyl transfer. Chem Rev 90: 1171-1202

Van Laere KMJ, Beldman G, Voragen AGJ (1997a) A new arabinofuranohydrolase from *Bifidobacterium adolescentis* able to remove arabinosyl residues from double-substituted xylose units in arabinoxylan. Appl Microbiol Biotechnol 47:231-235.

Van Laere KMJ, Bosveld M, Schols HA, Beldman G, Voragen AGJ (1997b) Fermentative degradation of plant cell wall derived oligosaccharides by intestinal bacteria. In: Hartemink R (ed) Non-digestible oligosaccharides: healthy food for the colon? Proceedings of the International

Symposium, Wageningen, The Netherlands, Wageningen Pres, Wageningen, The Netherlands, 37-46.

Valentine PJ, Gherardini FC, Salyers AA (1991) A *Bacteroides ovatus* Chromosomal locus which contains an α-galactosidase gene may be important for colonization of the gastrointestinal tract. Appl Envir Microbiol 57: 1615-1623

Weiser W, Lehmann J, Matsui H, Brewer CF, Hehre EJ (1992) Stereochemistry of the D-galactal and D-galacto-octenitol hydration of coffee bean α-galactosidase: insight into catalytic functioning of the enzyme. Arch Biochemistry Biophysics 292: 493-498

Zarate S, Lopez-Leiva MH (1990) Oligosaccharide formation during enzymatic lactose hydrolysis: a literature review. J Food Prot 53:262-268

# 6

# *Characterisation of a novel ß-galactosidase from Bifidobacterium adolescentis DSM 20083 active towards transgalactooligosaccharides*

This paper reports on the effects of both reducing and nonreducing transgalactooligosaccharides (TOS) comprising 2 to 8 residues on the growth of *Bifidobacterium adolescentis* DSM 20083 and on the production of a novel ß-galactosidase (β-Gal II). In cells grown on TOS, in addition to the lactose-degrading ß-galactosidase (ß-Gal I), another ß-galactosidase (ß-Gal II) was detected and it showed activity towards TOS but not towards lactose. ß-Gal II activity was at least 20-fold higher when cells were grown on TOS than when cells were grown on galactose, glucose, and lactose. Subsequently, the enzyme was purified from the cell extract of TOS-grown *Bi. adolescentis* by anion-exchange chromatography, adsorption chromatography and size-exclusion chromatography. ß-Gal II has apparent molecular masses of 350 and 89 kDa as judged by size-exclusion chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, respectively, indicating that the enzyme is active in vivo as a tetramer. ß-Gal II had an optimal activity at pH 6 and was not active below pH 5. Its optimum temperature was 35°C. The enzyme showed highest $V_{max}$ values towards galactooligosaccharides with a low degree of polymerisation. This result is in agreement with the observation that during fermentation of TOS the di- and trisaccharides were fermented first. β-Gal II was active towards ß-galactosyl residues that were 1→4, 1→6, 1→3, and 1↔1 linked, signifying its role in the metabolism of galactooligosaccharides by *Bi. adolescentis*.

## INTRODUCTION

The human colonic flora has both beneficial and pathogenic potential with respect to host health. There is now much interest in the manipulation of the microbiotic composition of the colon in order to improve the potentially beneficial effects (Gibson, 1998). Probiotics are live microbial supplements that beneficially affect the host by improving its intestinal microbial balance (Fooks et al, 1999; Fuller, 1989), and have been used for many years for this reason. As the viability of live bacteria in food products and during transit through the gastrointestinal tract may be variable, the concept of prebiotics has been developed. A prebiotic is considered to affect the host beneficially by selectively stimulating the growth and/or activity of one or a limited number of naturally present or introduced bacterial species in the colon. It has been claimed that this will also lead to an improvement in host health (Gibson and Roberfroid, 1995). Increasingly, probiotics and prebiotics are used in combination and are called synbiotics (Crittenden and Tannock, 1999; Fooks et la, 1999; Walker and Duffy, 1998). Fructooligosaccharides are considered prebiotics (Gibson, 1998) and oral dosages of transgalactooligosaccharides (TOS) appear to result in increased numbers of bifidobacteria in the human faecal flora (Bouhnik et al, 1997; Ito et al, 1990; 1993). It is claimed that a high number of bifidobacteria is beneficial for the health of the host. This may prevent colonisation by pathogens, and may have positive effects on intestinal peristalsis, on the immune system, in cancer prevention, on cholesterol metabolism, and on carbohydrate metabolism in the colon (Gibson and Roberfroid, 1995; Mitsuako, 1990).

TOS are oligosaccharides produced by transgalactosylation of lactose using a ß-galactosidase (ß-Gal). The linkage between the galactose units and the components in the final product depend on the enzymes and the conditions used in the reaction. The production and characterisation of these TOS have been described in various publications (Onishi and Tanaka, 1997; Prenosil et al, 1987; Smart, 1991). Different linkages between galactose and the reducing glucose unit have been identified, namely, ß-D-Gal$p$- (1→2)-D-Glc$p$, ß-D-Gal$p$-(1→3)-D-Glc$p$, ß-D-Gal$p$-(1→4)-D-Glc$p$ and ß-D-Gal$p$-(1→6)-D-Glc$p$. Also branched Glc$p$ residues occur, whereas oligogalactose fragments mainly contain 1→4 or 1→6 linkages (Smart, 1993; Zarate and lopez-Leiva, 1990). Recently, Fransen et al (1998) showed that nonreducing galactooligosaccharides were also formed during transgalactosylation of lactose.

Up to now no information has been available about the contribution of the various oligosaccharides in TOS to growth of *Bifidobacterium* species and nothing is known about their effect on the synthesis and activities of enzymes involved in oligosaccharide metabolism. Only artificial substrates, such as para-nitrophenyl β-D-galactoside, have been used to determine ß-Gal activities in bifidobacteria (Blanchette et al, 1992; Desjardin et al, 1990; Dumortier et al, 1994; Roy et al, 1994; Roy et al, 1992; Smart et al, 1993, Tochikura et al, 1986). However, this does not supply information about the number of enzymes involved and their specificities. Using classical culture methods and also molecular techniques it was shown that *Bifidobacterium adolescentis* is a major bifidobacterial species in the adult

intestinal microflora (Matsuki et al, 1999; Mutai and Tananka, 1987). In this paper we focus on the fermentation of various oligosaccharides in TOS by *Bi. adolescentis*. In addition to a lactose degrading β-Gal (β-Gal I), a novel ß-Gal (β-Gal II) involved in the degradation of TOS was purified and characterised. The role of the enzyme in the metabolism of TOS by *Bi. adolescentis* is discussed.

## MATERIALS AND METHODS

### Substrates

TOS (TOS) were obtained by transgalactosylation of lactose with a ß-Gal. The TOS mixture (Borculo Whey Products, Borculo, The Netherlands) was partially purified using charcoal chromatography in order to decrease the levels of mono- and disaccharides. The enriched oligosaccharide mixture contained 99% oligomers, next to some residual galactose, glucose, and lactose.

Enriched fractions containing [ß-D-Gal$p$-(1→6)]$_n$-D-Glc$p$ and [ß-D-Gal$p$-(1→4)]$_n$-D-Glc$p$ were obtained by fractionation of TOS from, respectively, Oligomate-50 (Yakult Pharmaceutical Co. Ltd.) and CUP-oligo (Nissin Sugar, Tokyo, Japan). Enriched fractions containing [ß-D-Gal$p$-(1→4)]$_n$-D-Gal$p$ (n=1-3) were obtained by incubation of extracted soy arabinogalactan with an endo-galactanase. After partial purification, their structure was confirmed using nuclear magnetic resonance NMR-spectroscopy. [ß-D-Gal$p$-(1→4)]$_n$-D-Glc$p$ (n=2-3) and ß-D-Gal$p$-(1→1)-D-Glc$p$ were purified and characterised as described by Fransen et al. (8). 3' Fucosyllactose, lacto-N-fucopentaose I, lacto-N-fucopentaose II, and ß-D-Gal$p$(1→6)-D-Gal$p$ were obtained from Dextra Laboratories Ltd. (Reading, United Kingdom), ß-D-Gal$p$(1→3)-Ara$f$ was obtained from ICN Biomedicals Inc., (Aurora, Ohio). $p$-Nitrophenyl (NP)-glycosides were obtained from Sigma (St. Louis, Mo.) or from Koch and Light, Ltd. (Haverhill, UK). Lactulose was obtained from Solvay (Weesp, The Netherlands). Melibiose was obtained from Jansens Chimica (Beerse, Belgium). Other chemicals were of analytical grade and obtained from commercial sources.

### Bacterial strain, culture conditions and oligosaccharide fermentation

*Bi. adolescentis* DSM 20083 was obtained from the Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH (Braunschweig, Germany). Cell extracts were prepared from *Bi. adolescentis* grown in M17 broth (Oxoid, Hampshire, England) for 48 h at 37°C in an anaerobic chamber with an atmosphere consisting of $CO_2$ (10%), $H_2$ (10%) and $N_2$ (80%). The pH of the medium was adjusted to pH 6.5 with KOH prior to sterilisation. Sugars (TOS, melibiose, lactose, galactose and glucose) (0.5% (w/v) of sugars in the M17 medium) were added from filter-sterilised stock solutions.

## Analysis of TOS

TOS from Borculo Whey Products, Yakult and Nissin were fractionated by Bio-Gel P-2 gel size-exclusion chromatography (100*2.6 cm), 200-400 mesh (Bio-Rad) with the column thermostated at 60°C, using a Pharmacia Hiload system equipped with a Pharmacia P50 pump. A Shodex RI-72 detector was used to monitor the refractive index of the water used as the eluent (0.3 ml/min). The oligosaccharide compositions of various fractions were established using high-performance anion-exchange chromatography (HPAEC). For this purpose a Dionex Bio-LC system (Sunnyvale, Califo.) that included a quaternary gradient pump, an eluent degas (He) module, and a 4 by 250 mm Carbopac PA100 column with matching guard column. Samples (20μl) were injected into the system using a Spectra Physics SP8800 autosampler (San Jose, Califo.) and chromatograms were recorded using a PC 1000 system. The sodium acetate gradient (1ml/min) in 100 mM NaOH was as follows: 0-30 min, linear gradient of 0-200 mM was used. The effluent was monitored using the PED detector containing a gold electrode with an Ag-AgCl reference electrode. The column was washed for 5 min with 1M NaOAc and equilibrated again for 15 min with 100mM NaOH before the next run was performed.

## Preparation of cell extracts

*Bi. adolescentis* DSM 20083 was grown as described previously, and the cells were harvested by centrifugation (10,000 x g, 10 min, 4°C) upon reaching the stationary phase. Cells were washed once in 20 mM phosphate buffer (pH 6.5) and then resuspended in 10 ml of the same buffer. Cells were disrupted on ice by sonic treatment (15 min, duty cycle 30%). Subsequently, the suspension was centrifuged at 10,000 x g for 10 min to remove non-disrupted cells and the resulting supernatant was centrifuged at 30,000 x g for 60 min to pellet cell-debris. The supernatants were filter sterilised and assayed for enzyme activity. The protein contents of the cell extracts were determined using the method of Bradford (Bradford, 1976) with bovine serum albumin as a standard.

## Enzyme assays

ß-Gal activity was measured by determining the hydrolysis of *p*-NP-ß-D-galactopyranoside (PNPG) at 40°C after 60 min of incubation. The reaction mixture (125 μl) contained 75 μl of 50 mM phosphate buffer (pH 6), 25 μl of 0.1% PNPG solution, and 25 μl of cell extract. The increase in absorbance (405 nm) was measured. A unit of enzyme activity was defined as 1 μmol of galactose liberated per min in 50 mM phosphate buffer (pH 6) at 40°C. The molar extinction coefficient under these assay conditions was 13700 $M^{-1}cm^{-1}$.

The hydrolytic activities of ß-Gal on TOS and the different oligosaccharides and polysaccharides were calculated from the amount of galactose released as determined by

HPAEC. The incubation was performed at 40°C for 1 h and the reaction mixture consisted of 100 µl of 0.1% (w/v) substrate in 50 mM phosphate buffer (pH 6).

## Production and purification of ß-Gal II

ß-Gal II was purified from the crude cell extract from *Bi. adolescentis* grown on 0.5% (w/v) TOS. Unless stated otherwise all procedures were carried out at room temperature. All buffers contained 0.01% (w/v) sodium azide to prevent microbial growth. Collected fractions were screened for protein content (A280 or the Sedmak method) and for ß-Gal activity.

The purification steps of the cell extract involved Bio-Gel HTP hydroxyapatite (Bio-Rad laboratories, Richmond), Q-Sepharose, Mono Q HR5/5, and Sephacryl S200 HR 16/60. The last three columns were from Pharmacia LKB Biotechnology, Uppsala, Sweden.

## pH and temperature optimum of ß-Gal II at the conditions used

The optimum temperature was determined by incubation of the ß-Gal with 0.1 % (w/v) TOS in 50 mM phosphate buffer pH 7 at 20, 30, 35, 40, 45, 50, 60 °C for 1h. The optimum pH was determined by incubating the ß-Gal with 0.1% (w/v) TOS in citrate-phosphate buffer in a pH range of 2.5 up to 8.0 for 1h at 40°C.

## Kinetic parameters of the ß-Gal II

Different substrates (PNPG or lactose or ß-D-Gal$p$-(1→4)-ß-D-Gal$p$-(1→4)-D-Glc$p$ or ß-D-Gal$p$-(1→4)-ß-D-Gal$p$-(1→4)-ß-D-Gal$p$-(1→4)-D-Glc$p$ or ß-D-Gal$p$-(1→4)-D-Gal$p$, ß-D-Gal$p$-(1→4)-ß-D-Gal$p$-(1→4)-D-Gal$p$ or ß-D-Gal$p$-(1→4)-ß-D-Gal$p$-(1→4)-ß-D-Gal$p$-(1→4)-D-Gal) with concentrations ranging from 0.1 to 30 mM in a 50 mM phosphate buffer (200 µl total volume, pH 6.0) were incubated with ß-Gal II (0.025µg protein/ 200 µl)) at 40°C for 1h. The $K_m$ and $k_{cat}$ values were calculated from the initial rates of the hydrolyses of oligosaccharides. Data analysis for calculation of kinetic parameters, using non-linear regression, was performed by the Enzfitter program (Biosoft, Cambridge, UK).

## Gel Electrophoresis

Native electrophoresis and SDS-PAGE electrophoresis were carried out with a PhastSystem (Pharmacia LKB Biotechnology, Uppsala, Sweden) according to the instructions of the supplier by using a 8 to 25% polyacrylamide gel or a 10 to 15% polyacrylamide gel (Pharmacia LKB Biotechnology).

Activity staining in acrylamide gels. Different cell extracts each containing 2 µg of protein were loaded and electrophoresed on a non-denaturing polyacrylamide gel electrophoresis system (Pharmacia LKB Biotechnology). ß-Gal activity was detected by incubating the gel in

a 4' umbelliferyl ß-galactoside solution (1mg/ml in 50 mM phosphate buffer pH 7). Fluorescent bands were visualised under UV light and photographed after incubation for 5 and 60 minutes.

## RESULTS

### Composition of TOS.

The TOS mixture was composed mainly of oligosaccharides (99 %) and small amounts of residual galactose (0.1%), glucose (0.3%), and lactose (0.6%). In order to determine the degrees of polymerisation of the oligosaccharides present, the oligosaccharide mixture was subjected to size-exclusion chromatography on a Bio-Gel P-2 column. Fractions corresponding to a given peak were pooled and subjected to HPAEC. In Figure 1 the HPAEC elution profile of the complete TOS mixture is given and the degree of polymerisation of the various peaks is indicated
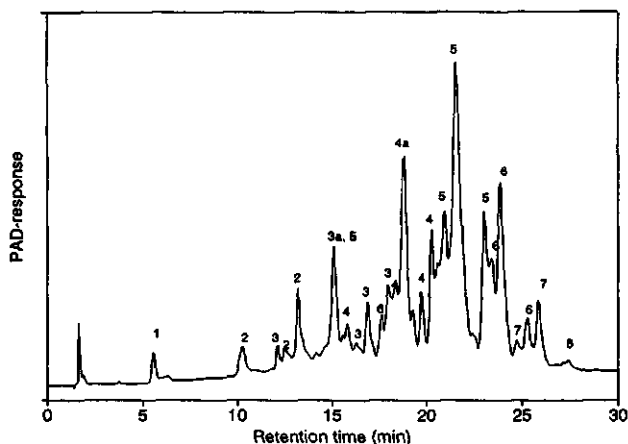


**Figure. 1.** HPAEC elution pattern of TOS. Numbers 1 to 8 indicate monomers, dimers, trimers, tetramers, pentamers, hexamers, heptamers and octamers respectively. Peaks 3a, 4a, and 5a have been identified as 4'galactosyllactose, 4'galactosyl galactosyl lactose and 4'galactosyl galactosyl galactosyl lactose, respectively. Note that peak areas do not supply information about the concentrations of the oligosaccharides since the response factor of the pulsed electrochemical detector varies significantly with the various oligomers.

All fractions were found to contain several components (data not shown) having the same degrees of polymerisation. The TOS mixture contains 0.5, 2, 6, 17, 37, 27, 8.5 and 2 % of mono-, di-, tri-, tetra-, penta-, hexa-, hepta-, and octamers, respectively as determined using a refractive index. The structures of the purified oligosaccharides are described elsewhere (8).

## Degradation of TOS by *Bi. adolescentis*

*Bi. adolescentis* grown in M17 containing glucose (0.5% w/v) (GM17) was transferred to fresh M17 medium (anaerobic, batch) containing 0.5 % (w/v) TOS. The growth (optical density) and acidification (pH) of the culture were measured (Figure 2) and residual TOS was analysed using HPAEC (Figure 3).
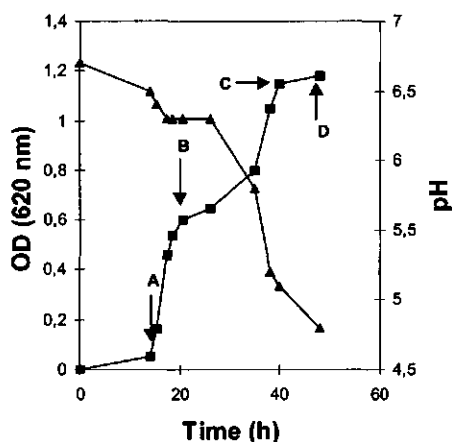
**I.**                                 **II.**
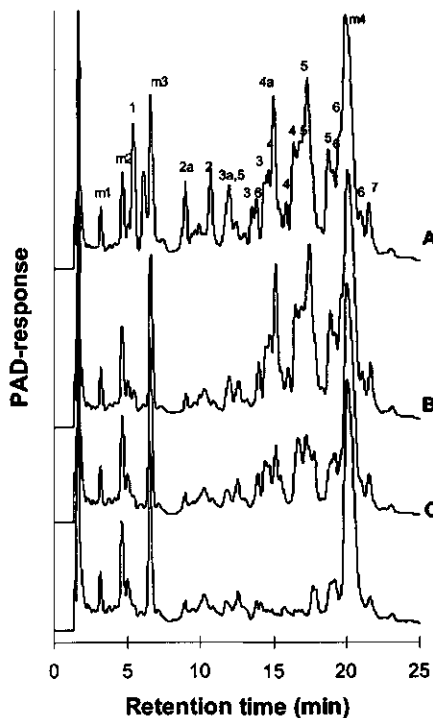


**Figure 2. I:**Growth of *Bi. adolescentis* in M17 containing 0.5% (w/v) TOS. Cells were precultured in M17 containing 0.5% (w/v) glucose. Arrows (A-D) indicate at which time samples were taken for HPAEC analysis of TOS in the supernatant

**II**: HPAEC elution pattern of TOS fermented by *Bi. adolescentis*. Samples were taken at 14h (A), 22 h (B), 40 h (C) and 50h (D) (see Fig 2). m1, m2, m3, and m4 are components present in M17 broth. Numbers 1 to 8 indicate monomers, dimers, trimers, tetramers, pentamers, hexamers, heptamers and octamers, respectively. Peaks 3a, 4a, and 5a have been identified as 4'galactosyllactose, 4'galactosyl galactosyl lactose and 4'galactosyl galactosyl galactosyl lactose, respectively.

After a lag-phase in which no carbohydrates were fermented (Fig 2.II.A), exponential growth occurred. At the beginning of this exponential growth-phase only monomeric material was fermented. At the end of the first exponential growth phase dimeric material and part of the trimeric material were fermented (Fig 2.II.B). Upon reaching an optical density of 0.6 a second lag-phase occurred. In the second exponential growth phase oligomers with a DP≥3

were fermented (Fig 2.II.C), with the low DP oligosaccharides being fermented first. Some residual TOS was observed after 50 h (Fig 2.II. D), which may have been due to inactivation of the enzyme at the low pH value reached at this point of fermentation (Fig 2.I.).

When *Bi. adolescentis* was precultured on TOS no biphasic growth could be observed and the stationary phase was reached within 30 h. These results suggests that cells have adapted to metabolise TOS more efficiently.

## Glycosidase activities in *Bi. adolescentis*

Subsequently, glycosidase activities in different cell extracts were determined with PNPG as a substrate. Specific ß-Gal activity was highest in cells grown on TOS (Table 1). Surprisingly, other glycosidase activities were also higher in TOS grown cells, including α-Gal, ß-glucosidase, ß-xylosidase and α-L-arabinofuranosidase. Although high levels of glycosidases were present, no endo-glycanase activity could be detected.

**Table 1:** Specific enzyme activities (mUnits/mg protein) in cell extracts from *Bi. adolescentis* DSM 20083 grown in the presence of different substrates.

| *p*-NP-glycosides | Sp Act | ((mU/mg) | of protein) | on growth | substrate: |
|---|---|---|---|---|---|
| | Gal*p* | Glc*p* | Lactose | TOS | Melibiose |
| *p*-NP-α-gal*p* | 530 | 995 | 745 | 2430 | 2220 |
| *p*-NP-ß-gal*p* | 65 | 155 | 40 | 1820 | 15 |
| *p*-NP-α-glc*p* | 10 | 270 | 30 | 100 | 900 |
| *p*-NP-ß-glc*p* | 240 | 270 | 200 | 1010 | 235 |
| *p*-NP-ß-xyl*p* | 60 | 80 | 55 | 300 | 75 |
| *p*-NP-α-ara*f* | 5 | 10 | 5 | 50 | 10 |

The activities of cell extracts from lactose- and TOS-grown cells towards TOS-pentamer were analysed by determining the release of galactose residues (Fig. 3). The amount of galactose released by the cell extract from TOS-grown cells was approximately 20-fold higher than that with cell extract of lactose-grown cells. Comparable low amounts of galactose were released also with cell extracts from galactose- and glucose-grown cells.
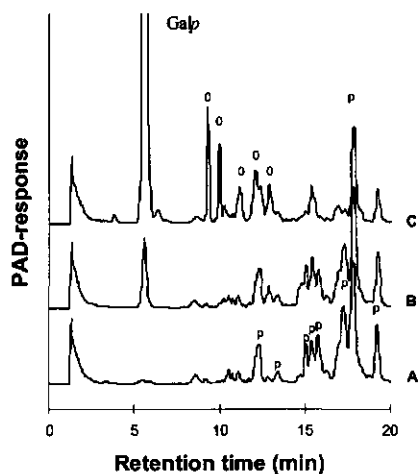
**Figure 3.** HPAEC elution patterns of galactopentasaccharides before (A) and after degradation (B and C) with the cell extracts of *B. adolescentis* grown on lactose (B) and on TOS (C). Gal*p*: galactose, p: pentamers, o: oligosaccharides formed by degradation of the galactopentasaccharides

β-Gal activities of cell extracts from galactose-, glucose-, lactose-, and TOS-grown *Bi. adolescentis* were also assayed after PAGE under nondenaturating conditions using 4'umbelliferyl ß-galactoside as substrate (Fig. 4).
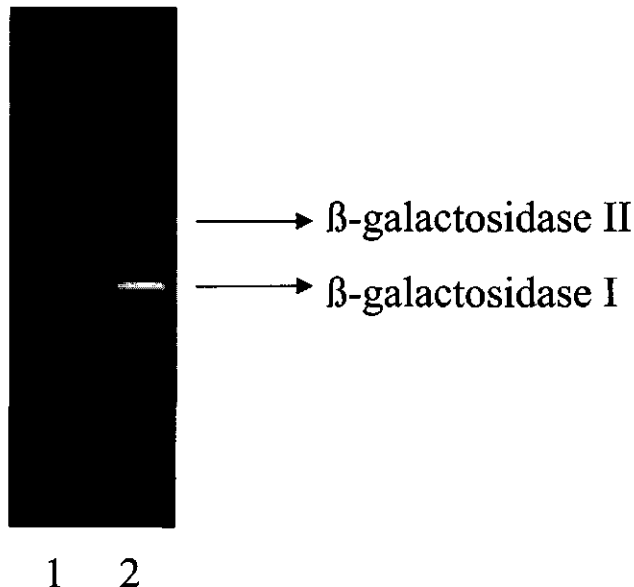


**Figure 4.** ß-Gal activity staining on a nondenaturing polyacrylamide gel. Crude enzyme preparations of *Bi. adolescentis* DSM 20083 grown on lactose (lane 1) and TOS (lane 2), with each preparation containing 2µg of protein, were supplied to the gel. ß-Gal activity was visualised under UV light after 5 min of incubation with a 4' umbelliferyl ß-galactoside solution.

In extracts from galactose-, glucose-, and lactose-grown cells one β-Gal band (β-Gal I) was observed, whereas in extracts from TOS grown cells, besides the increased β-Gal I band, a second β-Gal (β-Gal II) was detected. This second activity band was only faintly visible after prolonged incubation with cell extracts from galactose-, glucose-, and lactose-grown cells (data not shown), indicating that this enzyme is indeed present at very low levels in cells grown on sugars other than TOS (see below).

## Purification and characterisation of ß-Gal II

*Bi. adolescentis* was grown on M17 and TOS, and after reaching stationary phase, cells were harvested and the cell extract was prepared. No β-Gal activity could be detected in the culture supernatant. The cell extract contained 2 g of protein, and a total ß-Gal activity of 880 Units towards PNPG was measured. Cell extract from *Bi. adolescentis* was fractionated using Q-Sepharose, Bio-Gel HTP hydroxyapatite, Mono Q HR5/5, and Sephacryl S200 HR 16/60. The Q -Sepharose separation resulted in the separation of two different ß-Gal (ß-Gal I and ß-Gal II). The ß-Gal II-containing fraction showed activity towards TOS and no activity towards lactose, while the ß-Gal I-containing fraction was active towards lactose and not towards TOS. The TOS-active β-Gal II action was purified further on a Sephacryl S200 column and MonoQ column, and this resulted in an electrophoretically pure ß-Gal II preparation (not shown). The molecular mass was approximately 350 kDa, as estimated using Superose 12. By SDS-PAGE, a single protein band was found at 89 kDa suggesting that the enzyme is active as a tetramer *in vivo*.

## Substrate specificity and kinetic experiments with ß-Gal II

Subsequently, substrate specificity and physicochemical properties of β-Gal II were determined. Using PNPG the enzyme had a specific activity of 5.5 U/mg and optimal activity at pH 6 and 35°C. ß-Gal II was tested on a range of substrates. ß-Gal II was active towards the non-reducing oligosaccharide ß-D-Gal$p$-(1↔1)-D-Glc$p$ and to the other oligosaccharides present in the mixture including TOS with DP=8 (data not shown). Activity was also observed towards the galactooligosaccharides from other origin such as ß-D-Gal$p$-(1→3)-Ara$f$, ß-D-Gal$p$-(1→6)-D-Gal$p$, [ß-D-Gal$p$-(1→6)]$_n$-D-Glc$p$ with $n$=2-3 and [ß-D-Gal$p$-(1→4)]$_n$-D-Gal$p$ with n=1-3. The enzyme showed no detectable activity towards lactose, lactulose, 3' fucosyllactose, lacto-N-fucopentaose I, or lacto-N-fucopentaose II. Incubation of ß-Gal II in assays with increasing concentrations of ß1-4 linked galactooligosaccharides resulted in hyperbolic plots of substrate versus reaction rate, indicating typical Michaelis-Menten saturation kinetics. $K_m$-and $V_{max}$ values for the enzyme towards different galactooligosaccharides and PNPG are given in Table 2.

**Table 2:** Kinetic parameters of ß-Gal II from *Bi. adolescentis*

| Substrates | $K_m$ (mM) | $V_{max}$ (U/ml) | $V_{max}/K_m$ |
|---|---|---|---|
| PNPG | $2.2 \pm 0.2$ | $5.0 \pm 0.2$ | 2.3 |
| ß-D-Gal*p*-(1→4)-D-Glc*p* | 0 | 0 | - |
| ß-D-Gal*p*-(1→4)-ß-D-Gal*p*-(1→4)-D-Glc*p* | $2.2 \pm 0.2$ | $93.0 \pm 3.5$ | 42.2 |
| ß-D-Gal*p*-(1→4)-ß-D-Gal*p*-(1→4)-ß-D-Gal*p*-(1→4)-D-Glc*p* | $4.0 \pm 0.4$ | $68.1 \pm 3.2$ | 17.0 |
| ß-D-Gal*p*-(1→4)-D-Gal*p* | $3.7 \pm 0.3$ | $95.3 \pm 2.4$ | 25.7 |
| ß-D-Gal*p*-(1→4)-ß -D-Gal*p*-(1→4)-D-Gal*p* | $6.4 \pm 0.2$ | $48.6 \pm 0.5$ | 7.6 |
| ß-D-Gal*p*-(1→4)-ß -D-Gal*p*-(1→4)-ß -D-Gal*p*-(1→4)-D-Gal*p* | $5.2 \pm 0.7$ | $17.2 \pm 0.8$ | 3.3 |

These data clearly show a general decrease in the $V_{max}$ and catalytic efficiency (expressed as $V_{max}/K_m$) of ß-Gal II with an increase in the size of the oligosaccharides.

## DISCUSSION

This paper reports on the isolation and characterisation of a novel ß-Gal II from *Bifidobacterium adolescentis*, active towards TOS.

*Bi. adolescentis* produces two different ß-Gals when it is cultured on TOS. The first one (ß-Gal I), which was also detected in cells grown on glucose, galactose, and lactose, appeared to be active towards lactose, but not towards TOS. The TOS-active ß-Gal II was present at high levels only in TOS grown cells, indicating that synthesis is induced by the substrate. ß-Gal II was subsequently characterised further and was optimally active at pH 6 and 35°C, conditions mimicking those found in the colon. Remarkably, purified ß-Gal II showed no activity at pH 5 and below, which may affect the action of the enzyme in environments such as the colon where the pH may decrease below pH 6 due to microbial production of short chain fatty acids. ß-Gal II was active toward all the oligosaccharides present in the TOS mixture, including those with high DP. Kinetic characterisation of ß-Gal II revealed highest $V_{max}$–values towards oligosaccharides with low DP, which is in line with the sequential degradation observed during TOS fermentation. The ß-Gal II was also active towards ß-galactooligosaccharides derived from soy. However, ß-Gal II showed no activity towards fucosylated galactooligosaccharides isolated from human milk. The non-reducing disaccharide ß-D-Gal*p*-(1↔1)-D-Glc*p* isolated by Fransen et al. (1998) from the TOS mixture was completely hydrolysed into galactose and glucose monomers, indicating that these novel types of nonreducing oligosaccharides can be degraded by *Bi. adolescentis*.

Since microbial sugar transport systems described so far can take up monomers, dimers, or trimers (Poolman, 1993), it is speculated that ß-Gal II, in contrast to lactose-hydrolysing ß-Gal I, is located extracellularly. It is conceivable that the enzyme is cell wall or membrane attached, since no activity was found in the culture supernatant. With TOS, extracellularly released galactose residues from TOS by ß-Gal II would be accumulated via a galactose transport system and subsequently metabolised. The final remaining lactose may be taken up via a lactose transport system (Krezewinski et al, 1996 ; Poolman, 1993) and split intracellularly by ß-Gal I and the galactose and glucose residues may then metabolised. Several other *Bi. adolescentis* enzymes active towards large extracellular substrates were not found in the supernatant, which indicates that they are cell-wall or membrane bound (Van Laere et al, 1997; 1999). The recently characterised (Leder et al, 1999) and cloned (Broek van de et al, 1999) α-Gal, showing activity towards α-galactooligosaccharides, was shown to possess a signal sequence indicating that the enzyme is translocated to and active on the outside of the cell. Also, two arabinoxylan arabinofuranohydrolases from *Bi. adolescentis* were supposed to be membrane or cell wall associated and to degrade their substrate extracellularly, as they are active towards arabinose containing xylooligosaccharides which contain up to ten sugar units (Van Laere et al, 1997; 1999). Whether the binding of these glycosidases to the cell wall provides a competitive advantage e.g. in releasing substrates at the cell surface, remains to be elucidated.

The utilisation of the trisaccharides 4' galactosyllactose and 6' galactosyllactose by intestinal bacteria has been studied previously (Sako et al, 1999) and it has been shown that these substrates are not only fermented by bifidobacteria, but also by *Lactobacillus*, *Bacteroides* and *Clostridium* species. It is conceivable that these bacteria produce ß-Gals active towards these trisaccharides. So far the utilisation of galactooligosaccharides with a higher degree of polymerisation has not been reported. Our work shows that *Bi. adolescentis* can degrade also oligosaccharides with a DP >3 under these conditions. Other tested bacteria such as *Bifidobacterium infantis* and *Lactobacillus acidophilus* could utilise only the TOS with DP ≤ 3 present in the mixture (data not shown). Metabolism of high TOS by *Bi. adolescentis* is linked to the production of  ß-Gal II active towards these oligosaccharides. This enzyme might allow *Bi. adolescentis* to utilise the oligosaccharides more efficiently than other micro-organisms. Therefore, this TOS-mixture, containing mainly higher molecular weight material might be an interesting prebiotic substrate as it is metabolised by *Bi. adolescentis* being one of the predominant human faecal bacteria (Matsuki et al, 1999).

Strikingly, during growth of *Bi. adolescentis* on TOS a large number of glycosidases are produced, including two arabinofuranohydrolases which are involved in the degradation of arabinoxylooligosaccharides (Van Laere et al, 1997; 1999). This may offer an additional competitive advantage since it allows the organism to scavenge the environment for a range of substrates and use the degradation products for growth. No endo-glycanase activity could be detected in the cell extract (data not shown), suggesting that *Bi. adolescentis* adopted a strategy aimed at utilising polysaccharide degradation products generated by other

microorganisms instead of taking part in the initial depolymerization stage of polysaccharides.

The obtained results provide new insights in the oligosaccharide metabolism of bifidobacteria. Furthermore, the induction of ß-Gal II during growth on TOS indicates that optimal performance of a synbiotic product containing *Bi. adolescentis* (probiotic) and TOS (prebiotic) can be obtained by preculturing the microorganism on this substrate.

## REFERENCES

Blanchette DRL Savoie L, Ward P, Chevalier P (1992) Alpha- and beta-Galactosidase properties of *Bifidobacterium infantis*. Milchwissenschaft. 47:18-21

Bouhnik Y, Flourie B, D' Agay-Abensour L, Pochart P, Gramet G, Durand M, Rambaud JC (1997) Administration of transgalactooligosaccharides increases fecal bifidobacteria and modifies colonic fermentation metabolism in healthy humans. J Nutr 127:444-448

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt Biochem 72:248-254

Broek van den LAM, Ton J, Verdoes JC, Van Laere KMJ, Voragen AGJ, Beldman G (1999) Synthesis of α-galactooligosaccharides by a cloned α-galactosidase from *Bifidobacterium adolescentis*. Biotechnol Lett 21:441-445

Crittenden RG, Tannock GW(1999) Prebiotics. In: Probiotics: a critical review. GW Tannock (ed), Horizon scientific press, Wymondham, UK 141-156

Desjardins ML, Roy D, Goulet J (1990) Growth of bifidobacteria and their enzyme profiles. J Dairy Sci 73:299-307

Dumortier V, Brassart C, Bouquelet S (1994) Purification and properties of a beta-D-galactosidase from *Bifidobacterium bifidum* exhibiting a transgalactosylation reaction. Biotechnol Appl Biochem 19:341-354

Fransen, CTM, Van Laere KMJ, Wijk van AAC, Brüll LPB, Dignum M, Thomas-Oates JET, Haverkamp J, Schols HA, Voragen AGJ, Kamerling JP, Vliegenthart JGJ (1998) α-D-Glc$p$-(1↔1)-ß-D-Gal$p$-containing oligosaccharides, a new class of oligosaccharides produced by ß-galactosidase from lactose. Carbohydr Res 314:101-114

Fooks LJ, Fuller R, Gibson GR (1999) Prebiotics, probiotics and human gut microbiology. Inter Dairy J 9: 53-61

Fuller R (1989) Probiotics in man and animals. J Appl Bacteriol 66: 365-378

Gibson GR, Roberfroid MB (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics J Nutr 125:1401-1412

Gibson GR (1998) Dietary modulation of the human gut microflora using prebiotics. Br J Nutr 80: S209-S212

Ito M, Deguchi Y, Miyamori A, Matsuomote K, Kikuchi H, Matsumoto K, Yajima T, Kan T (1990) Effect of administration of galactooligosaccharides on the human faecal microflora, stool weight and abdominal sensation. Microb Ecol Health Dis 3:285-292

Ito M, Deguchi Y, Matsumoto K, Kimura M, Onodera N, Yajima T (1993) Influence of galactooligosaccharides on the human fecal microflora. J Nutr Sci Vitaminol 39:635-640

Krzewinski F, Brassart C, Gavini F, Bouquelet S (1996) Characterization of the lactose transport system in the strain *Bifidobacterium bifidum* DSM 20082. Curr Microbiol 32:301-307

Laere van KMJ, Voragen CHL, Kroef T, van den Broek LAM, Beldman G, Voragen AGJ(1999) Purification and mode of action of two different arabinoxylan arabinofuranohydrolases from *Bifidobacterium adolescen*tis. Appl Microbiol Biotechnol 51:606-613

Laere van KMJ, Beldman G, Voragen AGJ (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

Leder S, Hartmeier H, SP Marx (1999) α-Galactosidase of *Bifidobacterium adolescentis* DSM 20083 Curr Microbiol 38:101-106

Matsuki T, Watanabe K, Tanaka R, Fukuda M, Oyaizu H (1999) Distribution of bifidobacterial species in human intestinal microflora examined with 16 S rRNA-gene-targeted species-specific primers. Appl Environ Microbiol 65: 4506-4512

Mitsuoka T (1990) Bifidobacteria and their role in human health. J Ind Microbiol 6: 263-268

Mutai M, Tanaka R (1987) Ecology of *Bifidobacterium* in the human intestinal flora. Bifidobacteria Microflora 6:33-41

Onishi N, Tanaka T (1997) Purification and characterization of galactooligosaccharide-producing beta-galactosidase from *Sirobasidium magnum*. Lett Appl Microbiol 24:82-86

Poolman, B (1993) Energy transduction in lactic acid bacteria. FEMS Microbiol Rev 12:125-148

Prenosil JE, Stuker E, Bourne JR (1987) Formation of oligosaccharides during enzymatic lactose hydrolysis I. State of art. Biotechnol Bioeng 30:1019-1025

Roy D, Berger JL, Reuter G (1994) Characterization of dairy-related Bifidobacterium spp based on their beta-galactosidase electrophoretic patterns. Int J Food Microbiol 23:55-70

Roy D, Blanchette L, Savoie L, Ward P, Chevalier P (1992) alpha- and beta-Galactosidase properties of *Bifidobacterium infantis*. Milchwissenschaft 47:18-21

Sako T, Matsumoto K, Tanaka R (1999) Recent progress on research and applications of non-digestible galactooligosaccharides. Int Dairy J 9: 69-80

Smart JB (1991) Transferase reactions of the beta-galactosidase from *Streptococcus thermophilus*. Appl Microbiol Biotechnol 34: 495-501

Smart JB (1993) Transferase reactions of beta-galactosidases - new product opportunities. Bull Int Dairy Fed 289:16-22

Smart JB, Pillidge CJ, Garman JH (1993) Growth of lactic acid bacteria and bifidobacteria on lactose and lactose-related mono-, di- and trisaccharides and correlation with distribution of beta-galactosidase and phospho-beta-galactosidase. J Dairy Res 60:557-568

Tochikura T, Saka K, Fujiyoshi T, Tachiki T, Kumagai H (1986) *p*-Nitrophenyl glycoside-hydrolysing activities in Bifidobacteria and characterisation of ß-D-galactosidase of *Bifidobacterium longum* 401. Agric Biol Chem 50:2279-2286

Walker WA, Duffy LC (1998) Diet and bacterial colonization: role of probiotics and prebiotics. J Nutr Biochem 9:668-675

Zarate S, Lopez-Leiva MH (1990) Oligosaccharide formation during enzymatic lactose hydrolysis: a literature review. J Food Prot 53: 262-26

# 7

# *Fermentative degradation of hydrolysed arabinogalactan by intestinal bacteria*

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ß-Galactooligosaccharides (AGOS) were derived from soy arabinogalactan by enzymatic degradation. The derived mixture contained approximately 50% oligosaccharides with a degree of polymerisation (DP) ranging from 2-9, 20% of oligomers with a DP from 10-15 and 30 % of the total mixture had a degree of polymerisation>15.

The effect of AGOS on the growth of bacteria in faecal suspension was studied and compared to the effects of transgalactooligosaccharides (TOS). AGOS and TOS degrading strains belonging to the genera *Bifidobacterium* and *Bacteroides* could be isolated from faecal slurry after incubation of faecal inocula with these substrates. *Bi. adolescentis* and *B. vulgatus* were chosen as representatives for both genera to study the effect of TOS and AGOS on the regulation of ß-galactosidase. The highest levels of ß-galactosidase were present in the cell-extract (sI) containing the cell-associated and intracellular enzymes. Both substrates regulated the cell-bound oligosaccharide degrading activity of *Bi. adolescentis* and not of *B. vulgatus*. The oligosaccharide degrading activity of *B. vulgatus* was found to be of extracellular origin. TOS and AGOS both increased the galactan-degrading activity of *Bi. adolescentis*, whereas for *B. vulgatus* this increased activity towards polymeric galactan was only reached after growth on AGOS. These results suggest that growing *Bi. adolescentis* on TOS or AGOS would not only allow the bacteria to use the galactooligosaccharides, but would also allow more efficient utilisation of polymeric substrates as galactans, when both substrates are present together.

## INTRODUCTION

Transgalactooligosaccharides (TOS) are claimed to be prebiotic substrates predominantly utilised by bifidobacteria (Ito et al, 1990; 1993). They are produced by transgalactosylation of lactose using a ß-galactosidase (ß-Gal). Depending from the enzymes and the conditions used, structurally different galactooligosaccharides are obtained. The production and characterisation of TOS has been described in various publications (Onishi et al, 1997; Prenosil et al, 1987; Smart, 1991). Glycoside bonds between two galactose units are mainly ß1→4 bonds (4'-GOS) when ß-galactosidases derived from *Bacillus circulans* are used (Mozaffar et al, 1984), and ß1→6 bonds (6'-GOS) when enzymes derived from *Aspergillus oryzae* or *Streptococcus thermophilus* are used (Kimura et al, 1995). Different linkages between galactose and the reducing glucose unit have been identified, namely ß-D-Gal*p*-(1→2)-D-Glc*p*, ß-D-Gal*p*-(1→3)-D-Glc*p*, ß-D-Gal*p*-(1→4)-D-Glc*p* and ß-D-Gal*p*-(1→6)-D-Glc*p* (Smart, 1993; Zarate and Lopez-Leiva, 1990). Recently, Fransen et al (1998) showed that nonreducing galactooligosaccharides were also formed during transgalactosylation of lactose.

Bifidogenic effects have been demonstrated for different types of transgalactooligosaccharides in healthy adults (Tanaka et al, 1983; Sako, 1999). The aim of this study was to investigate whether other types of ß-galacto-oligosaccharides were also potential of interest as bifidogenic substrates, e.g. galactooligosaccharides produced by degradation of the plant cell wall polysaccharide arabinogalactan. Such arabinogalactans occur in two structurally different forms. Type I arabinogalactans are polysaccharides with a ß-1→4-linked backbone of galactopyranose residues substituted with α-arabinofuranose side chains of varying degree of polymerisation (Fig 1). The arabinose content of type I (arabino)galactan varies between 0% (w/w) for garlic (Das et al, 1977) to 43% (w/w) for soybean (Labavitch et al, 1976). Arabinogalactans have been isolated from various plants as reviewed by Clarke et al (1979).

→4)β-D-gal*p*-(1→4)β-D-gal*p*-(1→4)β-D-gal*p*-(1→4)β-D-gal*p*-(1→

α-L-araf-(1→5)- α-L-araf-(1→3)-

**Figure 1.** Proposed structure of type I arabinogalactan (Van de Vis, 1994)

Type II arabinogalactan is a highly branched polysaccharide with the basic feature of a branched chain of ß-D-galactopyranose residues, which is predominantly ß-1→3-linked in the interior backbone carrying to a varying degree 1→6-linked galactosyl residues. Type II arabinogalactans are found in flowering plants from various taxomomic group (e.g. *Larix* and *Acacia* species and rape seed) (Larm et al, 1976; Timell, 1965). A frequently used model for type II arabinogalactan is larch wood arabinogalactan (Clarke et al, 1979; van de Vis, 1994).

Several organisms produce galactanases that are specific for ß-1→4 galactopyranosyl linkages (Dekker and Richards, 1976; van de Vis, 1994) and therefore have the systematic name 1,4-ß-D-galactan galactanohydrolase (Dekker and Richards, 1976). This type of enzymes can be used for the production of ß-galactooligosaccharides, the structure of the oligosaccharides will depend on the type of enzyme and the incubation conditions used.

When added in our food, polymeric arabinogalactan is being fermented in the colon and mainly bifidobacteria and *Bacteroides* spp. have been shown to ferment this substrate (Van Laere et al, 2000; Crociani et al, 1994; Salyers et al, 1981). So far little research has focused on the fermentation of arabinogalactan derived oligosaccharides. Van Laere et al, (2000) showed that various micro-organisms including bifidobacteria and *Bacteroides* spp. could ferment arabinogalactooligosaccharides. However so far nothing is known about their behaviour in more complex faecal systems and their effect on the formation of enzymes involved in their degradation. The aim of this research was to establish the effect of AGOS and TOS on the growth of intestinal bacteria in faecal slurries and to study the influence of both substrates on the metabolic activity of some isolated strains.

## MATERIALS AND METHODS

### Materials and enzymes

Soy arabinogalactan was a gift from Novo Nordisk Ferment AG (Dittingen, Switzerland). Endo-galactanase was cloned from *Aspergillus aculeatus* (Kofod et al, 1995) and was kindly provided by Novo Nordisk A/S (Bagsvaerd, Denmark).

Linear ß-1→4 galactan was obtained by dearabinosylation of soy type I arabinogalactan using an arabinofuranosidase B from *Aspergillus niger* (Rombouts et al, 1988). Two different transgalactooligosaccharides mixtures (TOS$_1$ and TOS$_2$) were obtained by transgalactosylation of lactose with a ß-galactosidase and kindly provided by Borculo Whey Products (Borculo, The Netherlands). The TOS$_2$ mixture still contained 20 % monomers and 20% lactose whereas the TOS$_1$ was partially purified using charcoal chromatography in order to decrease the levels of mono- and disaccharides. The enriched oligosaccharide mixture contained 99% oligomers, besides to some residual galactose, glucose and lactose. The composition of both TOS-mixtures is given in Table 1.

## Production of arabinogalactooligosaccharides

A solution of arabinogalactan (15 g) consisting of 52% galactose and 38% arabinose in water (1500 ml) was digested with 15 mg cloned endo-galactanase for 17h at 40 °C. The incubation was stopped by heating (15 min, 100°C). The digest was concentrated by evaporation under reduced pressure and fractionated on a Sephadex G-10 column (800 × 100 mm) using the Äcta Explorer (Amersham Pharmacia Biotech, Uppsala, Sweden). Elution was carried out using water and the elution rate was 40 ml/min. The refractive index (Shodex RI-72) was monitored on-line. The molecular weight distribution was determined with BioGel P-2 column (1000 mm × 26 mm) (200-400 mesh, Biorad laboratories, Hercules, CA) at 60°C and eluted (20 ml/h) with distilled water. Some of the oligosaccharides present could be identified using standards present at the laboratory (Huisman et al, 2000).

## Preparation of stool samples

Stool samples were collected from 5 healthy volunteers. Samples were collected, diluted 10.000 fold using a physiological salt solution (rps) in an anaerobic chamber as described previously. Three further decimal dilutions were made in duplicate in carbohydrate free thioglycolate broth (made according to the formulation of Oxoid CM 173) supplemented with either 0.5% $TOS_2$ or AGOS. These dilutions were incubated anaerobically for 24 h. When growth was observed, 100 µl of these suspensions were subcultured into 900 µl of the same medium. This was repeated twice to enumerate bacteria capable of using $TOS_2$ and AGOS as selective substrate.

After three enumerations the samples were plated on semi-selective media to isolate predominant colonies. The media used were FRCA for total counts, RB for bifidobacteria, LAMVAB for lactobacilli (Hartemink and Rombouts, 1999), BBE for *Bacteroides fragilis* group (Summanen et al, 1993) and FRCA supplemented with vancomycin (20 mg/l) or colistin (50 mg/l) to suppress Gram positive and Gram negative organisms respectively.

All plates were incubated anaerobically for 48h at 37°C. After incubation $\sqrt{n}$ colonies were isolated and purified on FRCA medium. Isolates were further tested for growth on AGOS and $TOS_1$ in the medium described above. Isolates capable of fermenting these substrates were further characterised morphologically and biochemically according to Holdeman et al (1977).

## Organisms and culture media

*B. vulgatus* and *Bi. adolescentis* were precultured separately under anaerobic conditions in thioglycollate medium (Oxoid) containing 0.5% glucose at 37°C for 24-48 h. From these cultures 1 ml was added to 100 ml thioglycollate medium prepared according to the Oxoid formulation, without glucose but containing 0.5% (w/v) of respectively galactose, $TOS_1$ and AGOS. The bacteria were then cultured anaerobically for 24 h at 37°C. After the incubation the optical density (620 nm) and the pH using a micro-pH meter (Sentron, Roden, the

Netherlands) were measured. The degradation of the substrates after 24h of fermentation was monitored by High-Performance Anion-Exchange Chromatography (HPAEC). For these analysis the cultures were centrifuged and the supernatant was heated for 10 min at 100°C and frozen until further analysis.

## Enzyme-extract preparation and assay procedures

After 24h of fermentation the cells were harvested by centrifugation (10,000 x g, 10 min, 4°C). The supernatant (sE) was kept separately and contained the *extracellular enzymes*. To obtain the cell-extract (sI), the cells were washed once in 20 mM potassium phosphate buffer (pH 6.5) and then resuspended in 20 ml of the same buffer. Cells were disrupted by sonic treatment (15 min, duty cycle 30%) with a sonifier cell disrupter. During sonic treatment, the tube containing the cell suspension was kept on ice. Subsequently the suspension was centrifuged at 10,000 x g for 10 min, and the supernatant (sI) obtained after centrifugation of the cells was used as enzyme fraction containing both *intracellular and membrane or cell wall associated enzymes*. Both supernatants (sI and sE) were filter sterilised, 0.01% NaN$_3$ was added to prevent bacterial growth. Protein content of the crude enzyme extracts was determined using the method of Sedmak (Sedmak and Grossberg, 1977) with bovine serum albumine as a standard. ß-Gal activity was measured by determining the hydrolysis of *p*-NP-ß-D-galactopyranoside (PNPG) at 40°C after 60 min of incubation. The reaction mixture (125 µl) contained 75 µl of 50 mM phosphate buffer (pH 6), 25 µl of 0.1% PNPG solution, and 25 µl of cell extract. The increase in absorbance (405 nm) was measured. A unit of enzyme activity was defined as 1 µmol of galactose liberated per min in 50 mM phosphate buffer (pH 6) at 40°C. The molar extinction coefficient under these assay conditions was 13700 M$^{-1}$cm$^{-1}$. The degradation of TOS, AGOS and galactan were by enzymes present in the various extracts (sI and sE) was determined from the amount of galactose released as determined by HPAEC and HPSEC. The incubation was performed at 40°C for 4 h and the reaction mixture consisted of 200 µl of 0.5% (w/v) substrate in 50 mM phosphate buffer (pH 6,5).

## Analytical methods

The oligosaccharide composition of substrates and reaction mixtures was established using HPAEC. HPAEC was performed on a Dionex Bio-LC system (Sunnyvale, CA) as described by Schols et al, 1994). The gradient was obtained by mixing solutions of 0.1 M NaOH and 1 M sodium acetate in 0.1 M NaOH. For the determination of arabinogalactan oligomers, the (4*250 mm) CarboPac PA100 column (Dionex) was equilibrated with 0.1 M NaOH. Twenty µl of the sample was injected and a linear gradient to 0.4 M sodium acetate in 0.1 M NaOH in 40 min was applied. The column was washed for 5 min with 1 M sodium acetate in 0.1 M NaOH and equilibrated again for 15 min with 0.1 M NaOH.

Molecular weight distributions of the arabinogalactan treated by endo-galactanase were determined by High-Performance Size-Exclusion Chromatography (HPSEC), using three Bio-Gel TSK columns in series as described elsewhere (Bergmans et al, 1996).

## RESULTS

### Composition of the oligosaccharide mixtures

ß-Galactooligosaccharides were derived from soy type I arabinogalactan by enzymatic hydrolysis using an endo-1,4-galactanase. The mixture was partially purified using a Sephadex G-10 column in order to remove the contaminating monomeric and polymeric material. The degree of polymerisation of the groups of oligosaccharides making up the oligosaccharide mixture was estimated using Bio-Gel P2 (data not shown). The composition of the arabinogalactooligosaccharide (AGOS) mixtures as obtained is given in Table 1.

**Table 1:** Molecular weight distribution of the various mixtures (%)

| degree of polymerisation | AGOS | $TOS_1$ | $TOS_2$ |
|---|---|---|---|
| 1 | 1 | 0 | 18 |
| 2 | 5 | 3 | 40 |
| 3 | 10 | 6 | 22 |
| 4 | 17 | 17 | 14 |
| 5 | 5 | 37 | 5 |
| 6 | 4 | 26 | 1 |
| 7 | 4 | 9 | 0 |
| 8 | 3 | 2 | 0 |
| 9 | 4 | 0 | 0 |
| 10-15 | 18 | 0 | 0 |
| >15 | 28 | 0 | 0 |

Fractions containing oligosaccharides with similar DP were further separated using HPAEC (Fig 2) were partially identified using standards as described by Huisman et al (2000). Linear ß-D-(1→4)-linked galactooligosaccharides ($G_n$) with n=2-7 could be identified, besides some galactooligosaccharides containing arabinose ($G_nA$) or galactose side chains ($G_n$'). The HPAEC conditions used did not result in complete separation of all compounds present: especially at higher DP (>7) oligomers having different structures coeluted (Fig 2).
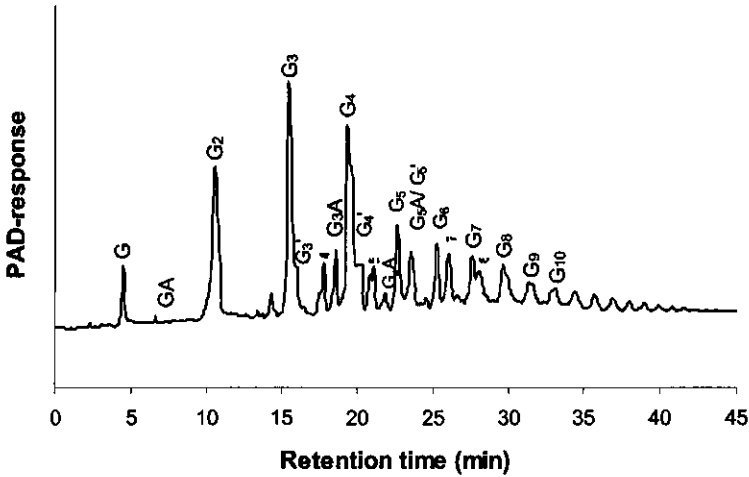
**Figure 2**: HPAEC Elution profile of the AGOS-mixture: G= Galactopyranose, A= Arabinofuranose.

## Characterisation of isolates utilising AGOS

The utilisation of AGOS and TOS by intestinal bacteria in a more complex system using faecal slurry was studied. After incubation of the faecal slurry with TOS or AGOS, the samples were plated on semi-selective media to isolate predominant colonies. Several morphologically different strains could be isolated from different plates and their morphological structure was microscopically examined. These included Gram positive and Gram negative straight rods and streptobacilli on FRCA, bifid shaped bacteria on RB and FRCA and pleomorphic rods on BBE. The isolated strains were cultured separately on TOS or AGOS for 48h and the fermentative degradation of the substrates after fermentation was monitored using HPAEC. After supplementation with TOS, bifidobacteria occurred in the highest dilution on FRCA and in the lower dilutions also *Bacteroides* strains and one *E .coli* was isolated. Addition of AGOS to the faecal slurry resulted in the growth of Bacteroides besides bifidobacteria on the FRCA plates. Streptobacilli were also present, however, none of the streptobacilli were capable of fermenting the two substrates, indicating growth on other components in the medium. No growth was observed on the LAMVAB plates. The strains fermenting AGOS were identified as *Bifidobacterium* species and *Bacteroides* species. When TOS was supplemented to the faecal slurries, an *E.coli* strain in addition to strains belonging to the genera *Bifidobacteria* and *Bacteroides* were identified as TOS-fermenting strains. Two strains were chosen for further research as representatives for both genera, one was identified as *Bifidobacterium adolescentis* and one as *Bacteroides vulgatus*.

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## Growth of *Bi. adolescentis* and *B. vulgatus* on TOS or AGOS

*Bi. adolescentis* and *B. vulgatus* were grown separately on galactose, TOS and AGOS. Both bacteria could utilise AGOS and TOS, and reached about the same optical density after 48 h of growth as after growth on the monosaccharide galactose (Table 2).

**Table 2:** Some characteristics of growth of *Bi. adolescentis* and *B. vulgatus* on different galactose containing carbohydrates (OD: optical density, DG: degradation ++ complete, + partially; PC: protein concentration of *sI* (mg/ml)

|  | Galactose | | | | TOS | | | | AGOS | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | OD | pH | DG | PC | OD | pH | DG | PC | OD | pH | DG | PC |
| *Bi. adolescentis* | 0.84 | 4.2 | ++ | 0.40 | 0.90 | 5.3 | ++ | 0.89 | 0.90 | 4.7 | ++ | 1.25 |
| *B. vulgatus* | 0.76 | 5.0 | + | 1.12 | 0.99 | 5.3 | + | 1.27 | 0.88 | 5.1 | + | 2.46 |

A clear drop in pH was observed compared to the blank (pH 6.5). The substrates were completely degraded by *Bi. adolescentis* and partially by *B. vulgatus* as monitored by HPAEC.

## Glycosidase activities of *Bi. adolescentis* and *B. vulgatus*

From each cultures, two different enzyme-extracts (sE and sI), were prepared to study the enzymes involved in the degradation of TOS and AGOS in more detail: one (sE), containing the extracellular enzymes and the other (sI) containing both membrane- or cell wall-associated and intracellular enzymes. The protein concentration was higher in the sI of *B. vulgatus* compared to the sI of *Bi. adolescentis* when grown on the same substrate (Table 2). ß-galactosidase activity towards *p*-NP-Gal was determined in the sE and sI of both *Bi. adolescentis* and *B. vulgatus* cultured on the three substrates (Table 3).

**Table 3:** ß-galactosidase activity towards *p*-NP-ß-galactoside present in the sE and sI from the various cultures.

| Bacteria (substrate in growth medium) | Total ß-galactosidase activity mU in sE | Total ß-galactosidase activity mU in sI |
|---|---|---|
| *Bi. adolescentis* (Galactose) | 70 | 16 |
| *Bi. adolescentis* (TOS) | 290 | 1900 |
| *Bi. adolescentis* (AGOS) | 250 | 2880 |
| *B. vulgatus* (Galactose) | 16 | 228 |
| *B. vulgatus* (TOS) | 160 | 180 |
| *B. vulgatus* (AGOS) | 180 | 1380 |

Growth on AGOS increased total ß-galactosidase activity for both *Bi. adolescentis* and *B. vulgatus* compared to galactose, with the highest activity present in the sI. The ß-galactosidase in the sI and the sE was also increased after growth of *Bi. adolescentis* on TOS. After incubation of TOS, AGOS and polymeric galactan with the various sI, degradation of the substrates was monitored by HPAEC. Growth of *Bi. adolescentis* on TOS and AGOS resulted in the regulation of galactose-releasing enzymes active towards TOS, arabinogalactooligosaccharides and polymeric galactan (Table 4). The degradation of AGOS by enzymes present in the sI of AGOS grown *Bi. adolescentis* cells is given in Fig 3.

**Table 4:** Degradability of TOS, the oligosaccharides in the AGOS-mixture and of polymeric ß1→4 galactan by the enzymes present in the sI of *Bi. adolescentis* and *B. vulgatus* sI as measured from galactose release. +: degradation, +- (*only* disaccharides were degraded), -: no degradation

| Bacteria (substrate in growth medium) | TOS | AGOS-mixture | Galactan |
|---|---|---|---|
| *Bi. adolescentis* (Galactose) | +- | +- | - |
| *Bi. adolescentis* (TOS) | + | + | + |
| *Bi. adolescentis* (AGOS) | + | + | + |
| *B. vulgatus* (Galactose) | - | - | - |
| *B. vulgatus* (TOS) | - | - | - |
| *B. vulgatus* (AGOS) | - | - | + |

Also the sE of *Bi. adolescentis* grown on AGOS, contained a ß-galactosidase which was active towards AGOS. However, it is not clear whether these glycosidases were secreted or released by senescent or lysed bacteria.
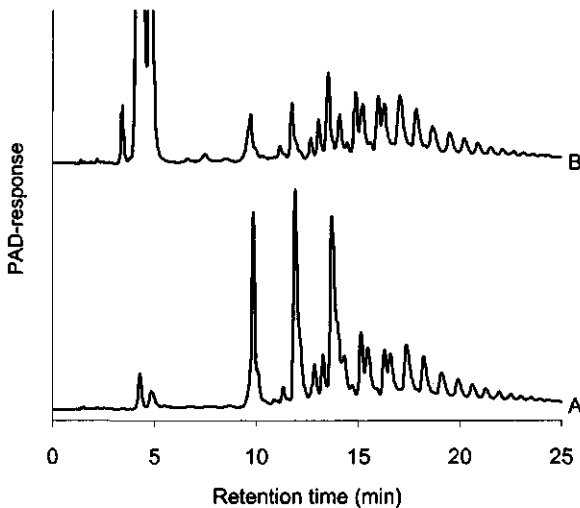


**Figure 3:** HPAEC elution profile of AGOS before (A) and after incubation with the sI from AGOS grown *Bi. adolescentis* (B).

Growth of *B. vulgatus* on TOS did not result in an increased ß-galactosidase activity in the sI compared to growth on galactose. In the sI of *B. vulgatus* grown on AGOS and TOS, no ß-galactosidase activity towards TOS and the oligosaccharides present in the AGOS-mixture could be detected (Table 4). The sE of *B. vulgatus* cultured on AGOS contained a ß-galactosidase active towards galactobiose, galactotriose and galactotetraose.

Incubation of AGOS with the sI of AGOS grown *B. vulgatus* resulted in the release of approximately 15% of the total galactose present. As the elution profile of the oligosaccharides in the mixture remained the same, the released galactose must originate from the polymeric part of the AGOS mixture. Polysaccharide degrading activity was confirmed, as in the sI also galactose-releasing activity was observed towards polymeric galactan. This increased polysaccharide degrading activity was not observed when *B. vulgatus* was grown on TOS.

## DISCUSSION

Bifidogenic effects of different types of transgalactooligosaccharides have been reported (Ito et al, 1993; Bouhnik et al, 1997; Tanaka et al, 1983). Often these oligosaccharide mixtures contain small oligosaccharides (average DP 3-4) which are though to be rapidly fermented in the proximal colon (Houdijk, 1998). This triggered us to look for other ß-galactooligosaccharides with higher DP, which might be fermented further in the colon. Therefore oligosaccharides were produced from soy arabinogalactan and their fermentation behaviour was compared to that of transgalactooligosaccharides.

Both oligosaccharide mixtures contain galactosyl residues as dominant building blocks in the backbone and these galactosyl residues are mainly ß-(1→4) linked. The oligosaccharides present in the AGOS-mixture may also contain arabinose residues (Huisman, 2000; Brüll, 1999). The TOS mixture contains some glucosyl residues and in addition to the ß-(1→4) linkages the presence of also ß-(1→2), ß-(1→3), ß-(1→6) and (1↔1) linkages has been reported (Fransen et al, 1998). The DP of TOS-mixture ranges from 2-8, whereas 50% (w/w) of the AGOS has a DP> 8.

These two substrates were compared with regard to their effect on growth of intestinal bacteria in faecal suspensions. Furthermore their effect on the production of ß-galactosidase activities by *Bi. adolescentis* and *B. vulgatus* was studied.

TOS an AGOS degrading strains belonging to the bifidobacteria were isolated from the highest dilutions of the faecal slurry cultured on AGOS and TOS. This shows that these strains have the capability to utilise both TOS and AGOS and to grow on them. In addition to the strains belonging to bifidobacteria, also some *Bacteroides* species were predominantly found in cultures supplemented with AGOS. TOS degrading strains belonging to the genus *Bacteroides* could also be isolated from the TOS-supplemented faecal slurry, however they occurred in lower levels. The higher levels of the *Bacteroides* strains in addition to the

bifidobacteria when AGOS was used as substrate compared to TOS could be explained by the presence of polymeric material in the AGOS mixture. Polymeric arabinogalactans are thought to be depolymerized by a relatively restricted group of anaerobic bacteria, chief among which are the *Bacteroides* spp and bifidobacteria. Arabinogalactan polysaccharides from larch wood (type II arabinogalactan) were utilised *in vitro* by *Bifidobacterium* spp. and *Bacteroides spp.* (Imamura et al, 1992; Crociani et al, 1994; Bayliss and Houston, 1984; Salyers et al, 1977). For TOS, on the other hand, bifidogenic effects have been demonstrated *in vivo* (Tanaka, 1983, Bouhnik, 1997). In studies with pure cultures it was shown that several bacteria including bifidobacteria, *Bacteroides* and some *E. coli* strains can degrade this substrate (Tanaka et al, 1983).

In more complex systems, factors such as competition for substrates between various strains, metabolic rates, presence of inhibitory substrates will determine the selection of bacteria that can finally profit of the substrate as energy source.

The utilisation of substrates by bacteria must be mediated by the hydrolysing enzymes they produce. *B. vulgatus* and *Bi. adolescentis* were chosen as model organisms for the *Bacteroides* group and the *Bifidobacterium* group respectively. *B. vulgatus* belongs to the *Bacteroides fragilis* group, and *Bacteroides fragilis*-type bacteria are among the most numerous groups of microorganisms that inhabit the human large intestine (Macfarlane and Gibson, 1991) They constitute between 20-30 % of the gut microflora (Holdeman et al, 1976; Salyers, 1984) with cell populations density typically being in excess of $10^{11}$ per gram (dry weight) content. *Bi. adolescentis* has been reported to be predominantly present in adult faeces (Matsuki et al, 1999; Mutai and Tanaka, 1987). Bifidobacteria account for up to 25% of the total cultivable gut flora of adults (Mitsuoka, 1984).

The total ß-galactosidase levels were generally highest in the sI, containing both cell-associated and intracellular enzymes, compared to the sE, containing the extracellular enzymes. This was found for both *B. vulgatus* and *Bi. adolescentis* independently on the substrate they were grown on. For species belonging to both genera it has been reported that most of the glycosidases are cell-bound (Berg, 1981; Bezkorovainy and Miller-Catchpole, 1989) and also *in vivo,* in the colon, the highest ß-galactosidase activity was cell-associated (Macfarlane et al, 1991). It is conceivable that the oligosaccharide degrading activity is cell wall or membrane attached, and not intracellular as it is thought that polysaccharides and oligosaccharides are initially degraded to smaller molecules (mono- and disaccharides) which can then be transported into the cell. Rarely, bacterial uptake systems are being reported for sugars with a degree of polymerisation > 2 (Russell et al, 1992).

Growth on TOS or AGOS did not increase the cell-bound oligosaccharide degrading activity of *B. vulgatus*. Using the AGOS mixture containing both oligomeric and polymeric material an increased polysaccharide degrading activity was observed for *B. vulgatus*. This is in agreement to what was found for the polymeric arabinogalactan as growth substrate. Various reports mentioned the increased cell-bound polysaccharide degrading activity when *Bacteroides* spp are grown on the arabinogalactan polymer (Macfarlane et al, 1990). When *B.*

*vulgatus* was grown on the pure oligomeric TOS no activity could be observed towards oligomeric and polymeric galactan. On the other hand growth of *Bi. adolescentis* on TOS and AGOS increased the levels of cell-bound enzymes involved in the degradation of oligomeric and polymeric galactan as compared to growth on galactose. In previous studies (Van Laere et al, 2000), it was shown that *Bi. adolescentes* only slightly degraded the arabinogalactan enriched polysaccharide type I fraction. Crociani et al, (1994) showed that *Bi. adolescentis* showed no activity towards type II arabinogalactan. Culturing *Bi. adolescentis* on a mixture containing galactooligosaccharides (TOS and AGOS) might enhance the degradation of polymeric galactan.

For *B. vulgatus* arabinogalactooligosaccharide degrading activity was observed in the extracellularly fraction. It can however be speculated that in more complex systems the presence of cell-bound glycosidases is advantageous for the bacteria, and allows them to transport the products of enzymatic breakdown immediately into the cells.

It can be concluded that both AGOS and TOS can be used by bifidobacteria as pure cultures but also in more complex systems. Both substrates seem to regulate the cell-associated galacto-oligosaccharide degrading activity of *Bi. adolescentis,* but not that of *B. vulgatus.* Both substrates also increased the galactan degrading activity of *Bi. adolescentis,* whereas this increased activity for *B. vulgatus* was only reached after growth on AGOS. These results suggest that growing *Bi. adolescentis* on TOS and AGOS would also allow the bacteria to more efficiently utilise polymeric substrates as galactans and thereby compete with other polysaccharide degrading bacteria for the substrate. The increased activity towards polymeric galactan after growth on TOS and AGOS might offer the bifidobacteria an additional advantage to compete for this polymeric substrate in the colon. Combining rapidly fermentable galactooligosaccharides with more complex galactose containing polymeric substrates might result in the availability of substrate for bifidobacteria over a larger part of the colon.

## REFERENCES

Bayliss CE, Houston AP (1984) Characterization of plant polysaccharide- and mucin-fermenting anaerobic bacteria from human feces. Appl Environ Microbiol 48: 626-632

Berg JO (1981) Cellular localization of glycoside hydrolases in *Bacteroides fragilis*. Curr Microbiol 5: 13-17

Bergmans MEF, Beldman G, Gruppen H, Voragen AGJ (1996) Optimisation of the selective extraction of (glucurono)arabinoxylans from wheat bran: use of barium and calcium hydroxide solution at elevated temperatures. J Cereal Sci 23: 235-245

Bezkorovainy A, Miller-Catchpole (1989) Biochemistry and physiology of Bifidobacteria. Bocca Raton: CRC Press

Bouhnik Y, Flourie B, D' Agay-Abensour L, Pochart P, Gramet G, Durand M, Rambaud JC (1997) Administration of transgalactooligosaccharides increases fecal bifidobacteria and modifies colonic fermentation metabolism in healthy humans. J Nutr 127: 444-448

Brüll, 1999 Mass spectrometric analysis of plant cell wall-derived oligosaccharides. PhD-thesis, University Utrecht, Utrecht, The Netherlands

Clarke AE, Anderson RL, Stone BA (1979) Form and functions of arabinogalactans and arabino-galactan-proteins. Phytochem 18: 521-540

Crociani F, Alessandrini A, Mucci MM, Biavati B (1994) Degradation of complex carbohydrates by *Bifidobacterium* spp. Int J Fd Microbiol 24: 199-210

Das NN, Das A, Mukherjee AK (1977) Structure of D-galactan isolated from garlic (*Allium sativa*) bulbs. Carbohydr Res 56: 337-349

Dekker RFH, Richards GN (1976) Hemicellulases: Their occurrence, purifications, properties, and mode of action. Adv Carbohydr Chem Biochem 32: 265-382

Fransen CTM, Van Laere KMJ, Wijk van AAC, Brüll LPB, Dignum M, Thomas-Oates JET, Haverkamp J, Schols HA, Voragen AGJ, Kamerling JP, Vliegenthart JFG (1998) $\alpha$-D-Glc$p$-(1$\leftrightarrow$1)-ß-D-Gal$p$-containing oligosaccharides, a new class of oligosaccharides produced by ß-galactosidase from lactose. Carbohydr Res 314: 101-114

Hartemink R, Rombouts FM (1999) Comparison of media for the detection of bifidobacteria, lactobacilli and total anaerobes from faecal samples. J Appl Bacteriol 36: 181-192

Holdeman LV, Cato EP, Moore WEC (1977) *Anaerobe Lab Manual.* Virginia Polytechnic Institute and State University, 4th edition, Blacksburg, Virginia, USA

Holdeman LV, Good IJ, Moore WEC (1976) Human fecal flora: a variation in bacterial composition within individuals and a possible effect of emotional stress. Appl Envir Microbiol 31: 359-375

Huisman M (2000) Elucidation of the chemical fine structure of polysaccharides from soybean and maize kernel cell walls. PhD-thesis, Wageningen University, Wageningen, the Netherlands

Houdijk J (1998) The effect of non-digestible oligosaccharides in young pig diets. PhD-thesis, Wageningen Agricultural University, Wageningen, the Netherlands

Imamura L, Murai K, Zhao C, Takebe S, Kobashi K (1992) Metabolism of arabinogalactan by rat and human intestinal microflora. BIFIDUS Flores, Fructus et Semina 6: 19-29

Ito M, Deguchi Y, Matsumoto K, Kimura M, Onodera N, Yajima T (1993) Influence of galactooligosaccharides on the human fecal microflora. J Nutr Sci Vitaminol 39: 635-640

Ito M, Deguchi Y, Miyamori A, Matsuomote K, Kikuchi H, Matsumoto K, Yajima T, Kan T (1990) Effect of administration of galactooligosaccharides on the human faecal microflora, stool weight and abdominal sensation. Microbiol Ecol Health Dis 3: 285-292

Kimura K, Matsumoto K, Ishihara C, Harada K, Miyagi A (1995) Structure determination of galacto-oligosaccharides by pyridylamination and NMR spectroscopy. Carbohydr Res 270: 33-42

Kofod LV, Mathiasen TE, Heldt-Hansen HP, Dalbøge H (1995) Application of cloned monocomponent carbohydrases for modification of plant materials. In: Petersen SB, Svensson

B, and Pedersen S (Eds) Progress in Biotechnology 10 Carbohydrate Bioengineering (321-342) Amsterdam, Elsevier

Labavitch JM, Freeman LE, Albersheim P (1976) Structure of plant cell walls: Purification of ß-(1-4)-galactanase which degrades a structural component of the primary cell wall of dicots. J Biol Chem 251: 5904-5910

Larm O, Theander O, Aman P (1976) Structural studies on a water-soluble arabinogalactan isolated from rape seed (*Brassica napus*). Acta Chem Scand 30: 627-630

Macfarlane GT, Hay S, Macfarlane S, Gibson GR (1990) Effect of different carbohydrates on growth, polysaccharidase and glycosidase production by *Bacteroides ovatus*, in batch and continuous cultures. J Appl Bacteriol 68: 179-187

MacFarlane GT, Gibson GR (1991) Co-utilization of polymerised carbon sources by *Bacteroides ovatus* grown in a two-stage continuous culture system. Appl Environ Microbiol 57: 1-6

Macfarlane GT, Gibson GR, Cummings JH (1991) Extracellular and cell-associated glycosidase activities in different regions of the human large intestine. Lett Appl Microbiol 12: 3-7

Matsuki T, Watanabe K, Tanaka R, Fukuda M, Oyaizu H (1999) Distribution of bifidobacterial species in human intestinal microflora examined with 16 S rRNA-gene-targeted species-specific primers. Appl. Environ. Microbiol. 65: 4506-4512.

Mitsuoka T (1984) Taxonomy and ecology of bifidobacteria. Bifid Microf 3: 11-28

Mozaffar Z, Nakanishi K, Matsuno R, Kamikubo T (1984) Purification and properties of beta-galactosidases from *Bacillus circulans*. Agric Biol Chem 48: 3053-3061

Mutai M, Tanaka R (1987) Ecology of *Bifidobacterium* in the human intestinal flora. Bifid Microf 6:33-41

Onishi N, Tanaka T (1997) Purification and characterization of galactooligosaccharide-producing beta-galactosidase from *Sirobasidium magnum*. Lett Appl Microbiol 24:82-86

Prenosil JE, Stuker E, Bourne JR (1987) Formation of oligosaccharides during enzymatic lactose hydrolysis. I. State of art. Biotechnol Bioeng 30:1019-1025

Rombouts FM, Voragen AGJ, Searle- Van Leeuwen MJF, Geraeds CCJM, Schols HA, Pilnik W (1988) The arabinanases of *Aspergillus niger*- Purification and characterization of two α-L-arabinofuranosidases and an endo-1,5-α-L-arabinanase. Carbohydr Polym 9: 25-47

Russell RR, Aduse-Opoku J, Sutcliffe IC, Tao L, Ferretti JJ (1992) A binding protein-dependent transport system in *Streptococcus mutans* responsible for multiple sugar metabolism. J Biol Chem. 5: 4631-7

Sako T, Matsumota K, Tanaka R (1999) Recent progress on research and applications of non-digestible galactooligosaccharides. Int Dairy J 9: 69-80

Salyers AA (1984) *Bacteroides* of the lower intestinal tract. Ann Rev Microbiol 38: 293-313

Salyers AA, Arthur R, Kuritza A (1981) Digestion of larch arabinogalactan by a strain of human colonic *Bacteroides* growing in continuous culture. J Agric Fd Chem 29: 475-80

Salyers AA, Vercelotti JR, West SEH, Wilkins TD (1977) Fermentation of mucus and plant polysaccharides by strains of *Bacteroides* from the human colon. Appl Envir Microbiol 37: 319-322

Schols HA, Voragen AGJ, Colquhoun IJ (1994) Isolation and characterization of rhamnogalacturonan oligomers, liberated during degradation of pectic hairy regions by rhamnogalacturonase. Carbohydr Res 256: 97-111

Sedmak JJ, Grossberg SE (1977) A rapid sensitive and versatile assay for protein using Commassie Brilliant Blue G250. Anal Biochem 79: 544-552

Smart JB (1991) Transferase reactions of the beta-galactosidase from *Streptococcus thermophilus*. Appl Microbiol Biotechnol 34: 495-501

Smart JB (1993) Transferase reactions of beta-galactosidases - new product opportunities. BIDFD 289:16-22

Summanen P, Baron EJ, Citron DM, Strong CA, Wexler HM, Finegold, SM (1993) *Wadsworth anaerobic bacteriol manual* 5th ed, Star Publishing Co, Belmont, USA

Tanaka R, Takayama H, Morotomi M, Kuroshim T, Ueyana S, Matsumot K, Kuroda A, Mutai M (1983) Effects of administration of TOS and *Bifidobacterium breve* 4006 on the human fecal flora. Bifidobacteria Microflora 2: 17-24

Timell TF (1965) Wood hemicelluloses: part II. Adv Carbohydr Chem Biochem 20: 409-477

Van Laere KMJ, Hartemink R, Bosveld M, Schols HA, Voragen AGJ (2000) Fermentation of plant cell wall derived polysaccharides and their corresponding oligosaccharides by intestinal bacteria. J Agric Fd Sc (in press)

Van de Vis JW (1994) Characterisation and mode of action of enzymes degrading galactan structures of arabinogalactans. PhD-thesis, Wageningen Agricultural University, Wageningen, the Netherlands

Zarate S, Lopez-Leiva MH (1990) Oligosaccharide formation during enzymatic lactose hydrolysis: a literature review. J Fd Prot 53:262-268

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# Fructooligosaccharides and transgalacto-oligosaccharides affect the bacterial glycosidase activity in human subjects

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Non-digestible oligosaccharides have been claimed to benefit the health of the colon by affecting composition and activity of the intestinal microflora. Two human trials to test whether fructooligosaccharides (FOS-study) and transgalactooligosaccharides (TOS-study) would change the bacterial glycosidase (particularly the ß-galactosidase) activity in the colon. In the FOS-study the fermentation of fructooligosaccharides in 24 healthy men by using a placebo-controlled crossover design (with glucose as the placebo) was investigated. Fructooligosaccharides significantly decreased $\alpha$-L-arabinofuranosidase activity by 35%. The TOS-study was a controlled feeding trial in which the effects of transgalactooligosaccharides were tested in 25 healthy volunteers receiving either transgalactooligosaccharides or placebo. Transgalactooligosaccharides significantly increased the activity of ß-galactosidase by 123%. Neither fructooligosaccharides nor transgalactooligosaccharides affected the activities of ß-glucosidase or ß-glucuronidase, which are enzymes involved in the generation of toxic compounds. It was concluded that TOS affect glycosidase activity of human intestinal bacteria in a substrate specific manner.

## INTRODUCTION

Non-digestible oligosaccharides naturally occur in various edible food plants (Loo *et al*, 1995) and in human breast milk (Carlson, 1985; Coppa *et al*, 1993; Grönberg *et al*, 1989). As they are not hydrolysed by the enzymes in the human small intestine, they reach the colon intact. Bacterial fermentation of oligosaccharides in the colon has been claimed to benefit its health by selectively stimulating the growth of bifidobacteria and by decreasing colon cancer risk markers (Ito *et al*, 1990; Bouhnik *et al*, 1997; Gibson *et al*, 1995*a*; Buddington *et al*, 1996; Bouhnik *et al*, 1996*b*).

Gut bacteria degrade non-digestible carbohydrates by using a wide range of depolymerizing enzymes. Polysaccharidase and glycosidase activities are found extracellularly as well as associated with bacterial cells (MacFarlane *et al*, 1991; Cummings and MacFarlane, 1991). By inducing these enzymes, dietary components may affect the metabolic activity of the microflora. Several studies were done to measure the end-products of bacterial breakdown of non-digestible oligosaccharides and often no effects were found on their faecal excretion (Alles *et al*, 1996; Alles *et al*, 1997*b*; Gibson *et al*, 1995*a*). This might be due to a rapid absorption and utilisation of fermentation products by the colonic mucosa (Höverstad *et al*, 1982; Cummings *et al*, 1987). MacFarlane *et al* (1991) measured the glycosidase activities in different regions of the human large intestine and conclude that activities were found throughout the large intestine without significant regional differences. Therefore, the activity of glycosidase in feces might be a more relevant measure to study the metabolic activity of the intestinal microflora, then faecal end-products of bacterial fermentation. Little is known about the glycosidase responses to consumption of non-digestible oligosaccharides by humans, but in a rat experiment by Djouzi *et al* (1997) it was found that there are profound differences between oligosaccharides.

We studied bacterial glycosidase activity induced by consumption of non-digestible oligosaccharides compared with digestible carbohydrates in two human trials. The first experiment was a free-living study done with fructooligosaccharides, which are short-chain fructans. The second experiment was a controlled feeding trial, using transgalactooligosaccharides, which are produced from lactose by enzymatic transgalactosylation. This work is an extension of previous studies that describes the fate of fructooligosaccharides in the human intestine (Alles et al, 1996) and the effects of transgalactooligosaccharides on microbial composition and colon cancer risk markers (Alles et al, 1999).

## METHODS

### Fructooligosaccharides study

Experimental methods are described in detail in Alles et al (1997). Briefly, twenty-four healthy men aged 19-28 years participated in a single-blind crossover trial with supplement periods of 7 d and a 7d wash-out period. The supplements where consumed in random order and consisted of either 15 g of fructooligosaccharides (Raftilose P95®, ORAFTI, Tienen, Belgium) or 4 g of glucose (Cerestar Pur 01934; Cerestar Benelux BV, Sas van Gent, The Netherlands). Fructooligosaccharides had a degree of polymerisation of 2-7 monosaccharide units.

At the end of each supplement period volunteers came to the Department twice to defecate. Within 15 min after defecation the feces were weighed and immediately deep-frozen on dry ice to stop fermentation, then stored at -20°C.

### Transgalactooligosaccharides study

This investigation formed part of a larger study, details of the experimental design and results on composition of the intestinal microflora and colon cancer risk markers are presented in Alles et al, 1999. The experiment was preceded by a screening procedure, comprising a healthy questionnaire, blood and urine analyses, a transgalactooligosaccharides tolerance test and a lactose breath test. Forty-one volunteers (19 women and 22 men) entered a strictly controlled single-blind parallel experiment. The study was divided into two consecutive 3-week periods during which each participant consumed a run-in diet in the first 3 weeks followed by a 3-week intervention diet which differed only in the amount of transgalactooligosaccharides: 0 g/d (placebo) or 15 g/d (Elix'or®, Borculo Whey Products, Borculo, the Netherlands). Transgalactooligosaccharides were produced by transgalactosylation of lactose and had a degree of polymerisation of 2-8 monosaccharide units. The diets consisted of conventional foods, and the nutrient composition of each diet was similar. Approximately 90% of energy intake was from supplied food, the remaining 10% was from products chosen by the subjects from a list of items. In the last week of both run-in and intervention period, volunteers came to the Department twice to defecate. Within 10 min after defecation the feces was deep-frozen on dry ice to stop fermentation, then stored at -20°C. In the last weekend of run-in and intervention periods, subjects collected all stools and stored them immediately on dry ice.

Both studies were approved by the Medical Ethics Committee of the Division of Human Nutrition and Epidemiology. The protocol and aims of the study were fully explained to the volunteers, who gave their informed written consent.

### Preparation of stool samples

Samples were thawed overnight at 4 °C. Feces of a single person of one period were weighted and homogenised in a bowl and mixer. A portion was used for the preparation of the aqueous

fraction of stool and centrifuged at 26000 g for 90 min at 4 °C (MSE, Scientific Instruments, Crawley, Sussex, UK). Faecal water was carefully removed and stored at -20°C until analysis of glycosidase activity.

## Bacterial glycosidase activities

Glycosidases are enzymes that can cleave off single units of sugar residues from glycosylated compounds including the non-reducing site of oligomeric sugars. The *p*-nitrophenyl glycoside assay (*p*-NP-assay) was used to measure the rate of release of *p*-nitrophenol from *p*-nitrophenylglycosides in faecal water. We tested the following bacterial glycosidase activities: α-L-arabinofuranosidase, α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, β-xylosidase, α-fucosidase. The reaction mixture contained 25 μl substrate solution (0.1% w/v, Sigma, St-Louis, MI) and 15 μl of the diluted faecal water (1:11) in 75 μl phosphate buffer (50 mM, pH 6.5). Incubation was at 37 °C and the release of *p*-nitrophenol from *p*-NP-glycosides was measured spectrophotometrically at 405 nm after addition of 125 μl of glycine (0.5 M glycine NaOH buffer, pH 9.0 + 2 mM EDTA). The activity was calculated using a molar extinction coefficient of 13700 $M^{-1}$ $cm^{-1}$. Incubation time changed between substrates. Enzyme activities were expressed as units, one unit corresponds to the release of 1 μmol glycoside $min^{-1}$ under standard conditions. Absorbances exceeding 1 after correction for the blank were diluted further and measured again.

## Statistical methods

Differences were checked for normality by visual inspection of the normal probability plots. For the fructooligosaccharides study, paired t-tests were used to test the difference between placebo and fructooligosaccharides supplementation. For the transgalactooligosaccharides study, unpaired t-tests were used to test the differences between changes. Two-sided *P*-values less then 0.05 were considered significant. The statistical analysis package SAS, version 6.09 (Statistical Analysis Systems Institute, Inc.; Cary, NC, USA) was used to perform the statistical analyses.

## RESULTS

### Fructooligosaccharides study

All volunteers completed the study successfully, were apparently healthy and had normal body weight (BMI 21.7 (SD 1.9) $kg/m^2$). In 4 faecal samples, there was too little faecal water extractable. Compared with the placebo treatment, there was a significant decrease (of 34.8%) in the arabinofuranosidase activity on the fructooligosaccharides supplement. There was a non-significant increase in ß-galactosidase activity of 45.3% (P = 0.0605) and a non-significant decrease in ß-xylosidase of 18.8% (P = 0.075). There were no other changes in glycosidase activity (table 1).

**Table 1**:Bacterial glycosidase activities (U/l faecal water) in 20 subjects receiving dietary supplements with 4 grams of glucose (placebo) or 15 grams of fructooligosaccharides in a cross-over design with supplement periods of 1 week[*]

|  | Placebo | | Fructooligosaccharides | | Difference (95% CI)[†] |
|---|---|---|---|---|---|
|  | Mean | SEM | Mean | SEM |  |
| α-L-Arabinofuranosidase | 400 | 61 | 261 | 30 | -139 (-256, -21)[‡] |
| α-Glucosidase | 221 | 23 | 189 | 16 | -32 (-81, 17) |
| β-Glucosidase | 133 | 9 | 113 | 11 | -21 (-49, 7) |
| α-Galactosidase | 328 | 86 | 244 | 65 | -84 (-292, 125) |
| β-Galactosidase | 373 | 42 | 542 | 94 | 169 (-8, 347) |
| β-Glucuronidase | 166 | 28 | 152 | 46 | -14 (-98, 70) |
| β-Xylosidase | 186 | 40 | 151 | 37 | -35 (-75, 4) |
| α-Fucosidase | 22 | 4 | 19 | 4 | -4 (-11, 4) |

α-L-Arabinofuranosidase, *EC* 3.2.1.55;
α-Glucosidase, *EC* 3.2.1.20;
β-Glucosidase, *EC* 3.2.1.21;
α-Galactosidase, *EC* 3.2.1.22;
β-Galactosidase, *EC* 3.2.1.23;
β-Glucuronidase, *EC* 3.2.1.31;
β-Xylosidase, 3.2.1.37;
α-Fucosidase, 3.2.1.51.

[*] Four observations were missing because there was too little faecal water extractable from feces.
[†] Fructooligosaccharides compared with placebo.
[‡] Difference between fructooligosaccharides and placebo was significant, $P = 0.0229$.

## Transgalactooligosaccharides study

One female subject withdrew in the first week of the experiment because of personal reasons; data from this subject were excluded from analyses. In four subjects, there was too little faecal water extractable from feces. All other subjects completed the study successfully. They were healthy and had normal body weight (BMI 22.8 (SD 2.3) kg/m$^2$).

Transgalactooligosaccharides significantly increased the activity of ß-galactosidase by 122.8%, after correction for placebo. There were no other changes in glycosidase activity (table 2).

**Table 2:** Bacterial glycosidase activities (U/l faecal water) in 21 subjects receiving a run-in diet for 3 weeks, and a placebo diet (with no transgalactooligosaccharides) or a diet with 15 grams of transgalactooligosaccharides for the subsequent 3 weeks[*]

| | Placebo (n=11) | | Transgalacto-oligosaccharides (n=10) | | Difference (95% CI)[†] |
|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | |
| α-L-Arabinofuranosidase | | | | | |
| Run-in | 398 | 49 | 254 | 61 | - |
| Intervention | 438 | 57 | 187 | 31 | -107 (-244, 29) |
| α-Glucosidase | | | | | |
| Run-in | 689 | 132 | 320 | 80 | - |
| Intervention | 657 | 140 | 486 | 264 | 198 (-244, 640) |
| β-Glucosidase | | | | | |
| Run-in | 45 | 12 | 35 | 8 | - |
| Intervention | 51 | 8 | 31 | 7 | -11 (-37, 16) |
| α-Galactosidase | | | | | |
| Run-in | 513 | 134 | 205 | 43 | - |
| Intervention | 447 | 96 | 215 | 34 | 76 (-136, 288) |
| β-Galactosidase | | | | | |
| Run-in | 787 | 121 | 474 | 53 | - |
| Intervention | 709 | 126 | 978 | 222 | 582 (141, 1022)[‡] |
| β-Glucuronidase | | | | | |
| Run-in | 87 | 20 | 57 | 12 | - |
| Intervention | 89 | 18 | 43 | 9 | -16 (-55, 23) |
| β-Xylosidase | | | | | |
| Run-in | 66 | 11 | 51 | 10 | - |
| Intervention | 69 | 9 | 47 | 9 | -8 (-30, 15) |
| α-Fucosidase | | | | | |
| Run-in | 29 | 8 | 19 | 4 | - |
| Intervention | 32 | 9 | 18 | 5 | -4 (-20, 11) |

Arabinofuranosidase, *EC* 3.2.1.55; α-glucosidase, *EC* 3.2.1.20; β-glucosidase, *EC* 3.2.1.21; α-galactosidase, *EC* 3.2.1.22; β-galactosidase, *EC* 3.2.1.23; β-glucuronidase, *EC* 3.2.1.31; β-xylosidase, 3.2.1.37; α-fucosidase, 3.2.1.51.

[*] Four observations were missing because there was too little fecal water extractable from feces.

[†] Transgalactooligosaccharides compared with placebo.

[‡] Difference between changes (intervention minus run-in) was significant, $P = 0.0124$.

## DISCUSSION

We found that transgalactooligosaccharides, affect the glycosidase activity of human intestinal bacteria in a substrate specific manner, without increasing those enzymes that are involved in the production of toxic and carcinogenic metabolites.

We measured the glycosidase activity aerobically in faecal water, which reflects the extracellular enzyme activity. As the feces was frozen before we prepared the faecal water, some intracellular enzymes might have been released due to lysis of bacteria. MacFarlane *et al* (MacFarlane *et al*, 1991) found that cell-bound activities are between 11 and 19 fold greater than those occurring extracellularly. The relative distribution of the enzymes however between extracellular and cell-bound enzymes did not differ much in their study.

Both the decrease of arabinofuranosidase on fructooligosaccharides and the increase of ß-galactosidase on transgalactooligosaccharides were not due to differences in the proportion of faecal wet weight. Correction for faecal wet weight did not change the results.

The availability of substrates to gut bacteria leads to the induction of substrate specific hydrolysing enzymes. Fructooligosaccharides can be hydrolysed by ß-fructosidase and transgalactooligosaccharides can be hydrolyzed by ß-galactosidase. Bouhnik *et al* (1996*b*) showed an increase in ß-fructosidase activity after consumption of fructooligosaccharides. In the TOS-study, we showed an enhanced activity of ß-galactosidase by specific substrate induction by transgalactooligosaccharides, which was not due to differences in faecal weight. This was also found by Djouzi *et al* (1997) in rats.

Substrates can also affect the activity of non-specific glycosidases enzymes, due to either changes in microflora composition or shifts in the metabolic activity of individual species or strains (Djouzi and Andrieux, 1997; Buddington *et al*, 1996; Reddy *et al*, 1992). We observed a decreasing effect of fructooligosaccharides on the activity of α-L-arabinofuranosidase, which was also not due to differences in faecal weight. Arabinofuranosidase is the enzyme that degrades arabinosyl linkages from various hemicelluloses such as arabinans, (arabino)xylans, (arabino)galactans and arabinose-substituted xyloglycans (Kaji, 1984). Fructooligosaccharides also increased the activity of ß-galactosidase and decreased the activity of ß-xylosidase. Probably due to the small number of volunteers relative to the large variation in enzyme activities, these changes were not statistically significant.

Changing the glycosidase activity may affect human health. Some of the enzymes help to generate energy for the colon mucosa in the form of butyrate. Increasing the activity of ß-galactosidase allows further degradation of lactose that escapes digestion and of arabinogalactans, which are important non-starch polysaccharides.

Other bacterial enzymes may have adverse effects in the colon. ß-Glucuronidase is involved in the hydrolysis of glucuronide conjugates in the gut, which lead to the generation of toxic and carcinogenic metabolites (Rowland, 1988; Mallett and Rowland, 1987). The hydrolytic activity of ß-glucosidase is responsible for the generation of mutagenic aglycones (Mallett and Rowland, 1987). We and others (Djouzi and Andrieux, 1997; Bouhnik *et al*, 1996*a*;

Kleessen *et al*, 1997; Bouhnik *et al*, 1996*b*) did not show any effects of fructooligosaccharides or transgalactooligosaccharides on the activity of ß-glycosidase and ß-glucuronidase. Buddington *et al* (1996) showed a decrease of ß-glucuronidase on fructooligosaccharides but did not compare the effects with a placebo treatment and was thus unable to exclude possible time effects.

This study shows that the glycosidase activity of human intestinal bacteria in feces can be used as a biomarker of colonic metabolic activity, in dietary interventions with non-digestible oligosaccharides. Although our study design was not ideally suited to test the differences between fructooligosaccharides and transgalactooligosaccharides, we showed that TOS affect the ß-galactosidase activity in a substrate specific manner. Both substrates did not affect those enzymes that generate toxic and carcinogenic metabolites.

## REFERENCES

Alles MS, Hautvast JGAJ, Nagengast FM, Hartemink R, Van Laere MKJ & Jansen JBMJ (1996) Fate of fructooligosaccharides in the human intestine. Brit J Nutr 76: 211-221

Alles MS , Katan MB., Salemans JMJI, Van Laere KMJ, Gerichhausen MJW, Rozendaal MJ, Nagengast FM (1997) Bacterial fermentation of fructooligosaccharides and of resistant starch in patients with an ileal pouch-anal anastomosis. Am J Clin Nutr 66: 1286-92

Alles MS, Hartemink R, Meyboom S, Harryvan JL, Van Laere KMJ, Nagengast FM, Hautvast JG (1999) Effect of transgalactooligosaccharides on the composition of the human intestinal microflora and on putative risk markers for colon cancer. Am J Clin Nutr 69: 980-991

Bouhnik Y, Flourié B, Andrieux C, Bisetti N, Briet F, Rambaud J-C (1996): Effects of Bifidobacterium sp fermented milk ingested with or without inulin on colonic bifidobacteria and enzymatic activities in healthy humans. Eur J Clin Nutr 50: 269-273

Bouhnik Y, Flourié B, D'Agay-Abensour L, Pochart P, Gramet G, Durand M, Rambaud J-C (1997): Administration of transgalactooligosaccharides increases fecal bifidobacteria and modifies colonic fermentation metabolism in healthy humans. J Nutr 127: 444-448

Bouhnik Y, Flourié B, Riottot M, Bisetti N, Gailing M-F, Guibert A, Bornet F, Rambaud J-C (1996): Effects of fructooligosaccharides ingestion on fecal bifidobacteria and selected metabolic indexes of colon carcinogenesis in healthy humans. Nutr Cancer 26: 21-29

Buddington RK, Williams CH, Chen S-C, Witherly SA (1996): Dietary supplement of neosugar alters the fecal flora and decreases activities of some reductive enzymes in human subjects. Am J Clin Nutr 63: 709-716

Carlson SE (1985): N-acetylneuraminic acid concentrations in human milk oligosaccharides and glycoproteins during lactation. Am J Clin Nutr 41: 720-726

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

Cummings JH, MacFarlane GT (1991): The control and consequences of bacterial fermentation in the human colon. J Appl Bacteriol 70: 443-459

Cummings JH, Pomare EW, Branch WJ, Naylor CPE, MacFarlane GT (1987): Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut 28: 1221-1227

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

Gibson GR, Beatty ER, Wang X, Cummings JH (1995): Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology 108: 975-982

Grönberg G, Lipniunas P, Lundgren T, Erlansson K, Lindh G, Nillson B (1989): Isolation of monosialylated oligosaccharides from human milk and structural analysis of three new compounds. Carbohydr Res 291: 261-278

Höverstad T, Böhmer T, Fausa O (1982): Absorption of short chain fatty acids from the human colon measured by the $^{14}CO_2$ breath test. Scand J Gastroenterol 17: 373-378

Ito M, Deguchi Y, Miyamori A, Matsumoto K, Kikuchi H, Kobayashi Y, Tajema Y, Kan T (1990): Effects of administration of galactooligosaccharides on the human faecal microflora, stool weight and abdominal sensation. Microb Ecol Health Dis 3: 285-292

Kaji A (1984): L-Arabinosidases. Adv Carbohydr Chem Biochem. 42: 383-394

Kleessen B, Sykura B, Zunft H-J, Blaut M (1997): Effects of inulin and lactose on fecal microflora, microbial activity, and bowel habit in elderly constipated persons. Am J Clin Nutr 65: 1397-1402

Loo JV, Coussement P, De Leenheer L, Hoebregs H, Smits G (1995): On the presence of inulin and oligofructose as natural ingredients in the western diet. Crit Rev Food Sci Nutr 35: 525-552

MacFarlane GT, Gibson GR, Cummings JH (1991): Extracellular and cell-associated glycosidase activities in different regions of the human large intestine. Lett Appl Microb 12: 3-7

Mallett AK, Rowland IR (1987): Dietary modification of intestinal bacterial enzyme activities - potential formation of toxic agents in the gut. Scand J Gastroenterol 129 (sup): 251-257

Reddy BS, Engle A, Simi B, Goldman M (1992): Effect of dietary fiber on colonic bacterial enzymes and bile acids in relation to colon cancer. Gastroenterology 102: 1475-1482

Rowland IR (1988): Factors affecting metabolic activity of the intestinal microflora. Drug Metab Rev 19: 243-261

# 9

## *General discussion*

## INTRODUCTION

### Aim of the research

The research described in this thesis was part of a multi-disciplinary research program at the Wageningen University on the role of non-digestible oligosaccharides in food and feed. Four groups were involved in the project: Human Nutrition, Animal Nutrition, Food Hygiene and Microbiology and Food Chemistry. The main objective of the research done at the Laboratory of Food Chemistry was to establish the relation between the chemical structure of non-digestible oligosaccharides and their fermentability by intestinal bacteria. For this, several structurally different oligosaccharides needed to be produced, isolated and characterised in order to obtain an oligosaccharide bank consisting of well-defined oligosaccharides. Secondly, the fermentation of the oligosaccharides by different intestinal bacteria was evaluated. This provided information on the fermentation capability of various intestinal species of bacteria. Thirdly, bacterial enzymes involved in the degradation of oligosaccharides were purified and characterised. This provided information on the regulation of their production, the activity of these enzymes and their specificity. Finally, we hoped to be able to design a tailor-made production of oligosaccharides using the knowledge obtained over the whole period.

## STRUCTURALLY DIFFERENT OLIGOSACCHARIDES OBTAINED FROM FOOD-GRADE RAW MATERIALS

In order to study structure-related effects of oligosaccharides on the fermentative degradation by intestinal bacteria, different oligosaccharides were produced. Using different routes and raw materials, oligosaccharides with different building blocks, degree of polymerisation, linkages and degree of branching were obtained. Fructooligosaccharides, xylooligosaccharides, transgalactooligosaccharides, and stachyose were obtained commercially. α-Galactooligosaccharides were obtained by transgalactosylation using α-galactosidase. Arabino-, (arabino)galacto-, (arabino)xylo-, galacturono-, and rhamnogalacturono- oligosaccharides were derived by enzymatic hydrolysis of plant polysaccharides. In table 1 the structurally different oligosaccharides studied in this thesis are given. Although not studied in this thesis, other potential valuable types of oligosaccharides are ß-gluco-, (galacto)manno-, and cellooligosaccharides obtained by hydrolysis of ß-glucans, galactomannans and celluloses, respectively.

Table 1: Oligosaccharides studied in this thesis (* commercially available)

| Type of oligosaccharide | Building block of backbone | linkage type in backbone | possible side chains | Enzyme used for production | Starting material |
|---|---|---|---|---|---|
| Fructooligosaccharides* DP 2-7 | Fructose, glucose | β-(2→1) | none | Inulinase | Chichory inulin |
| Xyloglucooligosaccharides | Glucose | β-(1→4) | xylose, galactose | Endo-glucanase | Tamarind seed xyloglucan |
| Transgalactooligosaccharides* | Galactose, glucose | β-(1→n) | none | β-Galactosidase | Lactose |
| Arabinogalactooligosaccharides | Galactose | β-(1→4) | arabinose, galactose | Endo-galactanase | Soy arabinogalactan |
| Stachyose* | Galactose, glucose, fructose | α-(1→6) β-(2→1) | none | None | Soy |
| 6' galactosyl melibiose | Galactose, glucose | α-(1→6) | none | α-Galactosidase | Melibiose |
| xylooligosaccharides* | Xylose | β-(1→4) | none | Endo-xylanase | Corn cobs, cotton husk bran |
| Arabinoxylooligosaccharides | Xylose | β-(1→4) | arabinose | Endo-xylanase | Wheat flour arabinoxylan |
| Arabinooligosaccharides | Arabinose | α-(1→5) | arabinose | Endo-arabinanase | Sugar beet arabinan |
| Galacturonooligosaccharides | Galacturonic acid | α-(1→4) | none | Polygalacturonase | Galacturonan |
| Rhamnogalacturonooligosaccharides | Rhamnose, Galacturonic acid | α-(1→4) α-(1→2) | galactose | Rhamnogalacturonan Hydrolase | Apple rhamnogalacturonan |

**New oligosaccharide structures using nature's complexity of plant cell wall polysaccharides**

The complex architecture of plant cell wall polysaccharides offers tremendous possibilities as starting material for producing structurally different oligosaccharides by enzymatic hydrolysis. The hydrolysis process can not only result in interesting potentially bifidogenic oligosaccharides, but partial hydrolysis might also result in increased solubility and changes in viscosity and may thereby facilitate the use or application of certain fibres.

Plant cell wall polysaccharides are natural components that are being used for years as dietary fibre in food and feed. In the colon, bacterial enzymes degrade dietary fibre and this can lead to the *in vivo* formation of oligosaccharides, disaccharides and monosaccharides. Plant cell wall derived oligosaccharides might therefore be regarded as naturally formed oligosaccharides. Furthermore, once they are formed they will be further degraded towards mono- and disaccharides, which shows that in the colon fermentation of plant cell wall derived oligosaccharide already occurs. However, it remains unknown which bacteria are involved in the utilisation of these substrates and one might assume that the kinetics and amount of oligosaccharides formed from fibre, and the place of fermentation in the colon will be different when these oligosaccharides are given directly as food supplements.

The oligosaccharides can be produced starting from pectins, hemicellulose and cellulose as they occur in plants. Many of the currently used fibres are insoluble or partly insoluble due to the fact that in the cell wall they are interconnected with other structural polysaccharides, proteins and possibly lignin. They often therefore, resist degradation and an initial extraction could be performed to make them accessible to enzymatic degradation. Pectins can be extracted using acid hydrolysis; however, these extraction processes are accompanied by inevitable and uncontrolled removal of neutral sugar side-chains such as arabinans and arabinogalactans (Voragen and Pilnik, 1995). Also, physical treatment (e.g. extrusion cooking and autoclaving) may improve the solubility of polysaccharides (Ralet et al, 1991; Guillon et al, 1992). Increasing the solubility of the polysaccharides will further improve the enzymatic degradation to oligosaccharides and can result in higher production yields. Galacturonooligosaccharides could be produced from the smooth regions of pectins. Arabinooligosaccharides, (arabino)-galactooligosaccharides and rhamnogalacturono-oligosaccharides were obtained from rhamnogalacturonan, a polysaccharide that is present in the cell walls of various plants. Both the rhamnogalacturonan backbone and the side-chains (galactan, arabinogalactans and arabinans) are rich in structurally different linkages and building blocks. This diversity allows the production of different oligosaccharides using specific endo-glycanases and debranching enzymes (Gruppen et al, 1992; Van Laere et al, 1994, Beldman et al, 1996; Beldman et al, 1997; Mutter, 1997). The arabinosyl and galactosyl side-chains described so far are relatively short, it would also be of interest to study the activity of these polysaccharides in combination with oligosaccharides derived from them.

One might assume that the intact polysaccharides will be fermented later in the colon because of their more complex structure.

Oligosaccharides, which were mainly composed of the pentose sugars arabinose and xylose were obtained from hemicellulose extracted from cereals. (Arabino)xylooligosaccharides were produced from wheat arabinoxylan but can also be produced from other cereals (corn, barley, rye, rice). The raw material source, extraction procedure and enzymatic degradation will influence the structure of the oligosaccharides produced. For the extraction of cereal hemicellulose polymers, NaOH and KOH are the most commonly used extractants that leave cellulose as residue (Brillouet and Mercier, 1981; Bergmans, 1996).

### Production of oligosaccharides using transglycosidase activity of glycosidase

Another method to obtain oligosaccharides is by transglycosylation using retaining glycosidases. Transglycosylation reaction can result in a complex mixture of structurally almost identical oligosaccharides. A typical example is the formation of transgalactooligosaccharides (TOS) from lactose using a bacterial ß-galactosidase (Figure 1). This resulted in the formation of at least 30 different oligosaccharides with a degree of polymerisation ranging from 2-8.
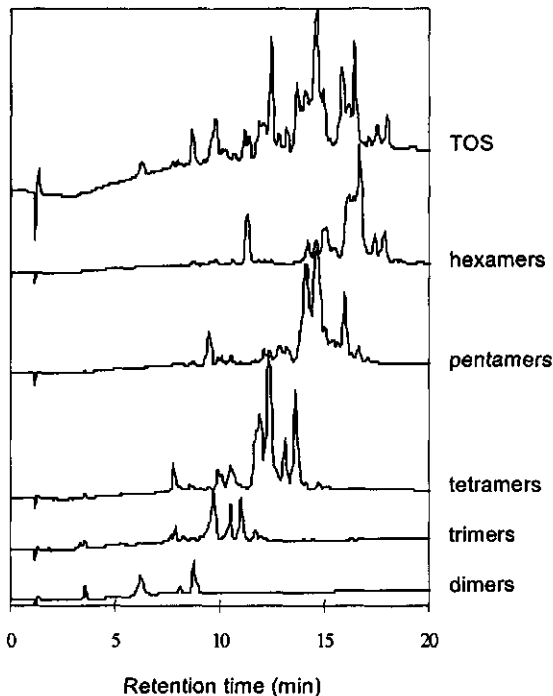


**Figure 1:** HPAEC-profiles of the starting TOS sample (upper panel, as obtained from Borculo Whey Products) and the different Bio-Gel P-2 fractions (disaccharides through hexasaccharides) (Fransen et al, 1998).

The structures of some of these transgalactooligosaccharides are already known, however, if complete characterisation is required, fractionation of the mixture is necessary. Separation on size (e.g. size-exclusion chromatography) can be used as the first step. Pools obtained after size-exclusion chromatography still contain mixtures of oligosaccharides having the same size. In Fig 1 the HPAEC elution profiles of the various pools are given.

Using HPAEC the oligosaccharides of the same size can be purified further. From this mixture the oligosaccharides ß-D-Gal$p$-(1→2)-Glc$p$, ß-D-Gal$p$-(1→3)-Glc$p$, ß-D-Gal$p$-(1→6)- ß-D-Gal$p$-(1→4)- Glc$p$ and (ß-D-Gal$p$-(1→4))n-Glc$p$ with n = 2-4 could be easily recognised. Also, twelve novel nonreducing oligosaccharides could be identified in this way, namely, (ß-D-Gal$p$-(1→4))n -α-Glc$p$-(1↔1)-ß-D-Gal$p$(-(4←1)-ß-D-Gal$p$)m with n, m = (0,1,2,3, or 4) and ß-D-Gal$p$-(1→2)-α-D-Glc$p$-(1↔1)-ß-D-Gal$p$. These non-reducing oligosaccharides have the advantage that they are not reactive in the Maillard reaction.

Some preliminary studies showed that also in the cell-extracts of *Bi. adolescentis* transgalactosylation activity towards lactose is present. This activity could not be contributed to the ß-Galactosidase II involved in the degradation of TOS. Upon reaction with lactose, various galactooligosaccharides were formed. Some of the oligosaccharides were coeluting using HPAEC with oligosaccharides formed by *Bacillus circulans* ß-galactosidase, which is used for the production of transgalactooligosaccharides. Mozaffar et al (1984) showed that using ß-galactosidases derived from *Bacillus circulans* mainly ß1→4 bonds between two galactose units are formed. However, in our study the structures of the oligosaccharides formed were not further elucidated. But it was clearly shown that various structures can be formed starting from lactose using *Bi. adolescentis* ß-galactosidase. These two examples clearly show that upon transferase reaction a multiplicity of structurally closely related oligosaccharides can be formed.
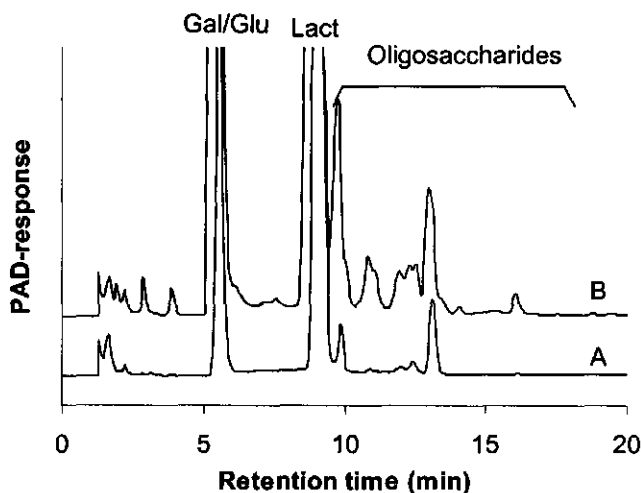


**Fig 2:** HPAEC elution profile of the transferase product obtained after incubation for 2h (A) and 24h (B) of lactose with the cell-extracts of *Bi. adolescentis*. Gal: galactose; Glu: glucose, Lact: lactose.

The variety of oligosaccharides obtained by transglycosylation reactions using glycosidases is limited by the availability of inexpensive starting material. Complex oligosaccharides can be produced starting from UDP-sugars in combination with glycosyltransferases. However, these UDP-sugars are expensive, and this approach is therefore limited for the preparation of oligosaccharides that are effective at low concentrations.

Various glycosidases possess besides their glycosidase activity also transglycosidase activity (McCarter and Wither, 1994). Recently, Pitson et al. (1996) showed that arabinofuranosidase A from *A. niger* is a retaining enzyme and is thus potentially valuable for the production of oligosaccharides by transglycosylation. A suitable substrate for this reaction (e.g. arabinobiose) can be obtained by extensive hydrolysis of arabinan using an endo-arabinanase. Some preliminary experiments showed that incubation with arabinobiose and this arabinofuranosidase A from *Aspergillus niger*, resulted in new types of arabinooligosaccharides (Fig 3). The oligosaccharides T1 and T2 coeluted with the arabinooligosaccharides derived from linear ($\alpha$1→5) arabinan (Fig 3, line A) and could therefore tentatively identified as $\alpha$-L-Araf-(1→5)-$\alpha$-L-Araf-(1→5)-L-Araf (T1) and $\alpha$-L-Araf-(1→5)-$\alpha$-L-Araf-(1→5)-$\alpha$-L-Araf-(1→5)-L-Araf (T2). Some unidentified structures (T3, T4, T5) are probably representing arabinooligosaccharides having other linkage types. The reaction conditions need to be further optimised to obtain higher yields and to establish the preferable acceptor glycosides of the enzyme.
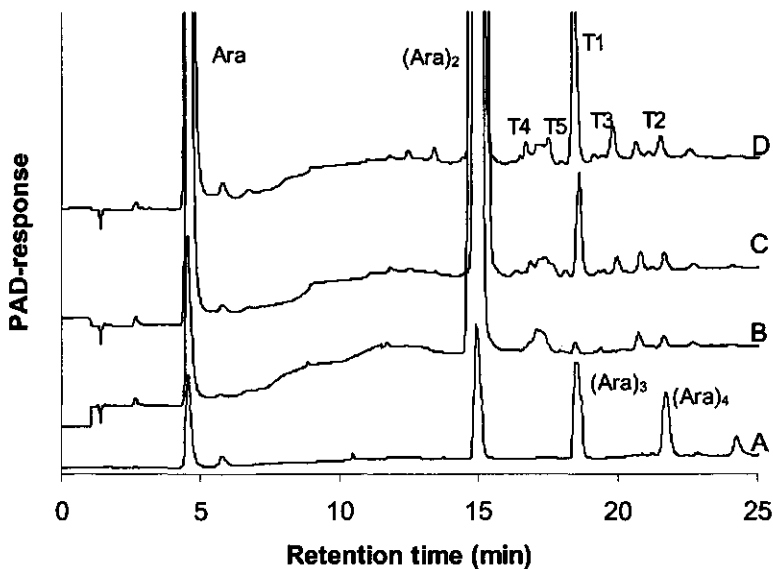


**Figure 3** : HPAEC-elution profile of arabinooligosaccharides produced by transglycosylation of arabinobiose using *A. niger* arabinofuronsidase A. Arabinobiose (10% w/v) was incubated with Arabinofuranosidase A (333 mU/ml) for 20 min ( C) and 1h ( D), respectively, at 40°C. A: endo-arabinase digest of sugar beet arabinan. B: arabinobiose (control). Samples were diluted 100 times prior to analysis on HPAEC. T1-5 are products formed during the transferase reaction.

These types of reactions offer tremendous possibilities for broadening the diversity of oligosaccharide structures. Although so far only applied to arabinooligosaccharides, comparable transglycosylation reaction could result in the production of mannooligosaccharides, xylooligosaccharides, cellooligosaccharides and galacturonooligosaccharides, starting from hydrolysed mannan, xylan, cellulose and pectin respectively in combination with the appropriate glycosidase. Whether these new-formed oligosaccharides behave physiological different remains to be studied. One could speculate that for intestinal degradation other bacterial glycosidases are necessary which could result in a different fermentation as from the starting oligosaccharide.

From this thesis it became clear that using enzymes and food-grade starting material a diversity of structures can be produced. However, the availability of reliable *in vitro* assays, predicting the physiological effects of these novel oligosaccharides, is essential to understand their structure-function relationship and will determine their tailored production in the future.


## UTILISATION OF OLIGOSACCHARIDES BY INTESTINAL BACTERIA

In this thesis, we describe the fermentative degradation of structurally different oligosaccharides by various intestinal bacteria. Our experiments provide information on the capability of pure cultures of intestinal species of bacteria to utilise the polysaccharides and oligosaccharides *in vitro*. Our study does not permit predictions about fermentation *in vivo*, since this will depend on various factors such as the availability of other substrates, growth factors, intestinal pH, actual number of bacteria and the interactions between the different species of bacteria present.

Bifidobacteria utilise various types of oligosaccharides *in vitro*. To do this, they need a wide range of glycosidases and a system to transport the small glycosyl moieties formed into the cell. Also, other bacteria are able to ferment oligosaccharides and *in vivo* competition for the substrate will occur. To test whether new types of oligosaccharides could be valuable sources, these oligosaccharides should be utilised -- at least *in vitro* -- by important beneficial bacteria. If these substrates are also utilised *in vitro* by other bacteria gives not prediction about the *in vivo* fermentation. However, oligosaccharides on which pathogenic bacteria flourish *in vitro* might already be avoided.

Species belonging to Bifidobacteria were able to ferment a wide range of oligosaccharides (Van Laere et al, 2000; Kontula et al, 1998; Jaskari et al, 1998): fructooligosaccharides, ß-galactooligosaccharides, α-galactooligosaccharides, (arabino)-galactooligosaccharides, (arabino)-xylooligosaccharides, arabinooligosaccharides, xylooligosaccharides and ß-glucooligosaccharides. The highly branched xyloglucooligosaccharides and the acidic galacturonooligosaccharides and rhamnogalacturonooligosaccharides which is both hghly branched and acidic, were not fermented by the bifidobacteria tested. Some individual species of bifidobacteria were also able to ferment polymeric material to some extent. Monitoring the

degradation revealed that it proceeds by an exo-mechanism (data not shown). This is in agreement with the literature. The presence of endo-glycanase in species of bifidobacteria was only mentioned in a few reports (e.g. amylase of *Bi. adolescentis* (Lee et al, 1997)). The fermentation of these different types of oligosaccharides by bifidobacteria shows that these bacteria produce different glycosylhydrolases. The purification and characterisation of the enzymes can provide interesting information on their specificity and mode of action.

## GLYCOSYLHYDROLASES OF *BI. ADOLESCENTIS*

High glycosidase levels measured with synthetic substrates such as *p*-NP- and 4-methylumbelliferyl conjugated substrates in enzyme assays have been reported for bifidobacteria. The patterns of these glycosidases have been suggested for use as a criteria for identification of bifidobacteria (Roy et al, 1994; O'Brien and Mitsuoka, 1991; Chevalier et al, 1992). Glycosidase activity to these synthetic substrates does not automatically mean oligosaccharide-degrading activity. To study whether glycosylhydrolases of intestinal bacteria are active towards oligosaccharides, the mode of action towards these substrates should be tested.

Using both classical culture methods and also molecular techniques it was shown that *Bifidobacterium adolescentis* is a major bifidobacterial species in the adult intestinal microflora (Matsuki et al, 1999; Mutai and Tanaka, 1987). Therefore this species of bifidobacteria was chosen as representative for the Genus *Bifidobacterium* and some of the glycosylhydrolases of *Bi. adolescentis* were studied in more detail.

### Arabinoxylan arabinofuranohydrolase

In this thesis, two novel glycosyl hydrolases of *Bi. adolescentis* (AXH-d3 and AXH-m2,3) are described. Both enzymes are specifically involved in the degradation of arabinoxylooligosaccharides and were called arabinoxylan arabinofuranohydrolases. These enzymes, in combination with a ß-xylosidase, allowed *Bi. adolescentis* to utilise arabinoxylooligosaccharides completely. The arabinose side-chains of xylooligosaccharides clearly influenced the fermentability by intestinal bacteria: linear xylooligosaccharides could be fermented by various species of intestinal bacteria, whereas double-substituted arabinoxylooligosaccharides could only be fermented by *Bacteroides vulgatus* and *Bi. adolescentis*. Whether similar enzymes are involved in the degradation of the double-substituted arabinoxylooligosaccharide by *B. vulgatus* remains unclear and needs further investigation. One of the enzymes, AXH-d3 was also active towards polymeric arabinoxylan and improved debranching of arabinoxylan in combination with other arabinofuranohydrolases. However, for complete debranching of highly substituted arabinoxylan such as arabinoxylan isolated from wheat bran, additional debranching enzymes are required (Van Laere et al, 1999).

The high specificity of both enzymes was remarkable, as these enzymes were only active towards arabinoxylan-derived structures. As *Bi. adolescentis* was also active towards arabinogalactan oligosaccharides, arabinooligosaccharides and *p*-NP-α-L-arabinofuranoside, other arabinofuranosidases should also be present in the enzyme extract.

### α-Galactosidase

Bifidobacteria contain high α-galactosidase activity and ß-galactosidase activity. Both α-galactooligosaccharides and ß-galactooligosaccharides are currently commercially available as prebiotics. These types of oligosaccharides can also be formed using glycosidases from intestinal bifidobacteria. *Bi. adolescentis* was found to produce high levels of α-galactosidase. This enzyme was purified, characterised and used in transglycosylation reactions. The α-galactosidase acted with retention of configuration releasing α-galactose from *p*-NP-Gal*p*. This hydrolysis probably operates using a double displacement mechanism, and is consistent with the observed glycosyl transferase activity. From this action, the trisaccharide α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-D-Glc*p* and the tetrasaccharide α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-D-Glc*p* could be identified. The purified α-galactosidase was cloned and showed also transferase activity towards melibiose (Van den Broek et al, 1999).

The structure of the oligosaccharide production the *Bi. adolescentis* glycosidases give information on the type of oligosaccharides formed. Whether this is linked to the oligosaccharides they preferentially use, remains to be studied. Leder et al (1999) reported that *Bi. adolescentis* α-galactosidase was most active towards α-1→3 galactosyl linkages, while during transferase reactions, predominately α-1→6 linkage types accumulated.

### ß-Galactosidase

*Bi. adolescentis* produced at least two ß-galactosidase upon growth on TOS. Besides a ß-galactosidase that was active towards lactose, a novel ß-galactosidase (ß-Gal II) was formed. The enzyme was active towards various galactooligosaccharides, but showed no activity towards lactose. Remarkably, the purified ß-Gal II showed no activity at pH 5 and below, which may affect the action of the enzyme in environments such as the colon where the pH may decrease below pH 6 due to microbial production of short chain fatty acids. Our study clearly shows that *Bi. adolescentis* produces at least two different ß-galactosidases. Both ß-galactosidase showed activity towards *p*-NP-ß-galactoside, although, they had a different specificity. Increased ß-galactosidase levels in cell-extracts can be the result of various enzymes, and should therefore not automatically indicate increased ß-galactooligosaccharide degrading activity. To monitor this, the activity to the substrates of interest (e.g. galactooligosaccharides and polysaccharides) needs to be studied.

## REGULATION OF ß-GALACTOSIDASE ACTIVITY

Microbial cells have a diversity of mechanisms by which they can regulate their activities in response to changes in their external environment. Regulation of the ß-galactosidase production was observed for *Bi. adolescentis*, when TOS was used as a carbon source. The exact mechanism involved in the regulation remains unknown. It was observed that the regulation was non-specific as also other glycosidases increased in the cell-associated enzyme fraction of *Bi. adolescentis*. It was shown that *Bi. adolescentis* is able to utilise glucose, galactose en lactose independently of the previous nutritional history of the culture (Van Laere et al, 2000). When the organism was grown in the presence of glucose and subsequently transferred to a medium containing TOS, it was observed that the bacteria initially utilised the monomeric and dimeric material The large oligosaccharides were only used when the monomers and dimers were no longer available. This result manifested as diauxic growth. It was also shown that a new ß-galactosidase (ß-gal II) was formed when cultured on TOS and the enzyme showed activity against TOS but not to lactose.

In human volunteers a regulation of ß-galactosidase activity was also observed by supplementation of TOS (chapter 8). The glycosidase activity was measured aerobically in faecal water as a reflection of the extracellular enzyme activity, and not in the enzyme-fraction representing the cell-associated enzymes. However, as the faeces was frozen before preparation of faecal water, cell-associated and intracellular enzymes might have been released as a result of lysis of the bacteria. It would be interesting to establish what the origin is of the increased ß-galactosidase activity. In the future, monoclonal antibodies of ß-Gal I or ß-Gal II of *Bi. adolescentis* could be used to visualise if the increased ß-galactosidase activity could be linked to ß-galactosidase activity from *Bi. adolescentis*.

Whether higher levels of ß-galactosidase activities are beneficial remains unknown. The fact that these glycosidases are still higher in the faeces indicates that the enzymes could also be active in the colon and even in the distal colon. Although the mode of action of the ß-galactosidase found in the faecal water is not known, this ß-galactosidase might be helpful in the fermentative degradation of oligosaccharides and polysaccharides and thereby providing butyrate as energy source for the colon mucosa. Some publications also mention a decreased faecal ß-galactosidase activity in patients with active Crohn's disease (Favier et al, 1997) compared to healthy volunteers. Whether this difference in ß-galactosidase activity is physiologically relevant remains to be established. It would be interesting to study if supplementation of TOS to these patients would lead to increased levels of ß-galactosidase and to establish the consequences of increased levels of ß-galactosidase on the symptoms of the disease.

## SUPPLEMENTATION WITH POLYMERIC AND OLIGOMERIC GALACTANS

TOS and FOS were already fermented in the cecum (beginning of the large intestine) of piglets and it was suggested that larger and more complex oligosaccharides might result in stimulation of the growth of bifidobacteria over a larger part of the colon (Houdijk, 1998).

A mixture containing both oligomeric and polymeric material (DP>10) was produced by hydrolysis of soybean arabinogalactan (AGOS). Fermentation of this substrate in an *in vitro* fermentation model using faecal slurry resulted in the predominance of strains belonging to *Bifidobacterium* and *Bacteroides*. The strains belonging to *Bifidobacterium* fermented the oligosaccharides present in the mixture completely while the *Bacteroides* strains only partially fermented the galactooligosaccharides.

Galacto-oligosaccharide and -polysaccharide degrading enzymes were present in the enzyme-extract when *Bi. adolescentis,* as representive for the genus *Bifidobacterium*, was grown on AGOS These enzymes were also observed if *Bi. adolescentis* was grown on TOS, containing only oligosaccharides. When *B. vulgatus* as representatives for the genus *Bacteroides,* was grown on AGOS, only enzymes involved in the degradation of the polymeric part were present in the cell-extract. These enzymes were not produced by *B. vulgatus* when grown on TOS. The enzymes involved in the degradation of the galactooligosaccharides were produced extracellularly by *B. vulgatus*. This information suggests that *Bi. adolescentis* produced enzymes to degrade both polymeric and oligomeric galactan when grown on pure galactooligosaccharides and on a combination of galacto-oligosaccharides and -polysaccharides. *B. vulgatus* only produces these enzymes when grown on the combination of both polymeric and oligomeric galactan. How these substrates will be fermented *in vivo* remains unknown, however, one might presume that the location of the enzymes is important. It is probably advantageous for the bacteria to have these enzyme closely associated to the cell so that the products of enzymatic breakdown of oligosaccharides and polysaccharides can be transported immediately. Whether the combination of both ß-galactooligosaccharides and polymeric galactan will result in fermentation through the whole colon remains to be investigated. Other approaches might also be used to produce a shift of fermentation from the proximal to the distal part of the colon. Combination with other substrates can influence the site of fermentation. Govers et al, (1999), showed that wheat bran can influence the fermentation site of resistant starch. Possibly other fibres could produce a similar effect on the fermentation sites of the oligosaccharides.

## VIEW ON THE FUTURAL USE OF OLIGOSACCHARIDES IN FOOD AND FEED

- Oligosaccharides with varying degree of polymerisation and branching can be obtained by enzymatic modification of plant polysaccharides. Using different polysaccharides, oligosaccharides having different building blocks can also be produced. The availability

of the oligosaccharides will depend on the susceptibility of the parent polysaccharide to enzymatic hydrolysis

- During transglycosylation reactions, complex mixtures of oligosaccharides having different degree of polymerisation and linkage can be formed. Especially the variation in linkages between building blocks is an important feature in the production of new oligosaccharides. Extensive hydrolysis of polysaccharides may result in new acceptor or donor substrates, which can be used as starting material for further transferase reactions.

- Information about the fermentation of the different oligosaccharides provides insight about the capability of different species of bacteria to utilise the substrate as a pure culture. Our studies showed that in addition to bifidobacteria, *Bacteroides* and other bacteria also possess enzymes which are active towards the tested oligosaccharides.

- Using enzymes and food-grade starting material a diversity of structures can be produced. However, the availability of reliable *in vitro* assays, predicting the physiological effects of these novel oligosaccharides, is essential to understand their structure-function relationship and will determine their tailored production in the future.

- Monoclonal antibodies towards glycosylhydrolasees of intestinal bacteria (e.g. ß-galactosidase II of *Bi. adolescentis*) might be of use as biomarkers for the activity levels of the intestinal bacteria.

- Upon fermentation of (arabino)-xylooligosaccharides, two novel arabinoxylan arabinofuranohydrolases were produced. These enzymes *in* combination with a ß-xylosidase permitted *Bi. adolescentis* to utilise arabinoxylooligosaccharides *in vitro*. Therefore these arabinoxylooligosaccharides, which could be produced from various cereals, are interesting as potentially bifidogenic factors.

- α-Galactooligosaccharides having various linkages and polymerisation degree, produced by transgalactosylation of melibiose or raffinose using α-galactosidase, might be new bifidogenic oligosaccharides, as bifidobacteria have high α-galactosidase activity.

- Culturing *Bi. adolescentis* on TOS leads to an increased ß-galactosidase activity towards TOS. Administration of TOS in combination with probiotics (bifidobacteria and lactobacilli) precultured on TOS might result in a better survival or implantation of the probiotics in the gastrointestinal tract (synbiotic).

- Various oligosaccharides could be used by probiotic strains and might support the viability of the probiotic strains in the gastrointestinal tract.

- Strains from bifidobacteria and bacteroides were able to grow on a mixture consisting of both polymeric and oligomeric material. *Bi. adolescentis* produced cell-associated enzymes which were active towards both polymeric and oligomeric galactan while *B. vulgatus* only had cell-associated activity towards the polymeric fraction. Combinations of oligomeric with polymeric material (e.g. galactans), consisting of the similar building blocks and linkage types, might stimulate the utilisation of both substrates by

bifidobacteria. Whether this will result in a stimulation of bifidobacteria over a larger part of the colon remains to be studied.

## REFERENCES

Bergmans MEF, Beldman G, Gruppen H, Voragen AGJ (1996) Optimisation of the selective extraction of (glucurono)arabinoxylans from wheat bran: use of barium and calcium hydroxide solution at elevated temperatures. J Cereal Science 23: 235-245

Beldman G, Mutter M, Searle-van Leeuwen MJF, Broek van den LAM, Schols HA, Voragen AGJ (1996) New enzymes active towards pectic structures. In: Visser J and Voragen AGJ (eds), Pectin and pectinases. Progress in Biotechnol 14: 231-245, Elsevier, Amsterdam, The Netherlands

Beldman G, Schols HA, Pitson SM, Searle-van Leeuwen MJF, Voragen AGJ (1997) Arabinans and arabinan degrading enzymes. In: Sturgeon RG (ed) Advances in Macromolecular Carbohydrate Research 1: 1-64 JAI Press Inc

Brillouet JM, Mercier C (1981) Fractionation of wheat bran carbohydrates. J Sci Food Agric 32 : 243-251

Chevalier P, Roy D, Ward P (1990) Detection of *Bifidobacterium* species by enzymatic methods. J Appl Bacteriol 68: 619-624

Dumortier V, Brassart C, Bouquelet S (1994) Purification and properties of a beta-D-galactosidase from *Bifidobacterium bifidum* exhibiting a transgalactosylation reaction. Biotechnol Appl Biochem 19: 341-354

Favier C, Neut C, Mizon C, Cortot A, Colombel JF, Mizon J (1997) Faecal beta-D-galactosidase production and Bifidobacteria are decreased in Crohn's disease. Dig Dis Sci 42: 817-822

Fransen CTM, Laere KMJ van, Wijk AAC van, Brull LP, Dignum M, Thomas Oates JE, Haverkamp J, Schols HA, Voragen AGJ, Kamerling JP, Vliegenthart JFG (1998) alpha-D-Glc*p*-(1↔1)-beta-Gal*p*-containing oligosaccharides, novel products from lactose by the action of beta-galactosidase. Carbohydr Res 314: 101-114

Gruppen H, Hoffmann RA, Kormelink FJM, Voragen AGJ, Kamerling JP, Vliegenthart JFG (1992) Characterisation by $^1$H-NMR spectroscopy of enzymically-derived oligosaccharides from alkali-extractable wheat flour arabinoxylan. Carbohydr Res 233: 45-64

Govers MJAP, Gannon NJ, Dunshea FR, Gibson PR, Muir JG (1999) Wheat bran affects the site of fermentation of resistant starch and luminal indexes related to colon cancer risk: a study in pigs. Gut 45: 840-847

Guillon F, Barry JL, Thibault JF (1992) Effect of autoclaving sugar-beet fibre on its physico-chemical properties and its *in-vitro* degradation by human faecal bacteria. J Sci Food Agric 60: 69-79

Houdijk J (1998) Effects of non-digestible oligosaccharides in young pigs. PhD-thesis, Wageningen Agricultural University, Wageningen, The Netherlands

Jaskari J, Kontula P, Siitonen A, Jousimies-Somer H, Mattila-Sandholm T, Poutanen K (1998) Oat beta-glucan and xylan hydrolysates as selective substrates for Bifidobacterium and Lactobacillus strains. Appl Microbiol Biotechnol 49: 175-181

Kontula P, Wright A von, Mattila-Sandholm T (1998) Oat bran beta-gluco- and xylooligosaccharides as fermentative substrates for lactic acid bacteria. Inter J Food Microbiology 45: 163-169

Leder S, Hartmeier W, Marx SP (1999) α-Galactosidase of *Bifidobacterium adolescentis* DSM 20083. Curr Microbiol 38: 101-106

Lee SK, Kim YB, Ji GE (1997) Note: purification of amylase secreted from *Bifidobacterium adolescentis*. J Appl Microbiol. 83: 267-272

Matsuki T, Watanabe K, Tanaka R, Fukuda M, Oyaizu H (1999). Distribution of bifidobacterial species in human intestinal microflroa examined with 16 S rRNA-gene-targeted species-specific primers. Appl Environ Microbiol 65: 4506-4512

McCarter JD, Wither SG (1994) Mechanisms of enzymatic glycoside hydrolysis. Curr Opinion Struct Biol 4: 885-892

Mozaffar Z, Nakanishi K, Matsuno R, Kamikubo T (1984) Purification and properties of beta-galactosidases from *Bacillus circulans*. Agric Biol Chem 48: 3053-3061

Mutai M, Tanaka R (1987) Ecology of *Bifidobacterium* in the human intestinal flora. Bifidobacteria Microflora 6:33-41

Mutter M (1997) New rhamnogalacturonan degrading enzymes from *Aspergillus aculeatus*. PhD-thesis, Wageningen Agricultural University, Wageningen, The Netherlands

O'Brien M, Mitsuoka T (1991) Quantitative fluorometric assay for rapid enzymatic characterization of *Bifidobacterium longum* and related bifidobacteria. Microbiol Immunol 35: 1041-1047

Pitson SM, Voragen AGJ, G Beldman (1996) Stereochemical course of hydrolysis catalyzed by arabinofuranosyl hydrolases. FEBS Lett 398: 7-11

Ralet MC, Thibault JF, Della Valle G (1991) Solubilization of sugar-beet pulp cell wall polysaccharides by extrusion-cooking. Lebensm- Wiss Technol 24: 107-112

Roy D, Berger JL, Reuter G (1994) Characterization of dairy-related *Bifidobacterium spp.* based on their beta-galactosidase electrophoretic patterns. Inter J Food Microbiol 23: 55-70

Van den Broek LAM, Ton J, Verdoes JC, Van Laere KMJ, Voragen AGJ, Beldman G (1999) Synthesis of alpha-galactooligosaccharides by a cloned alpha-galactosidase from *Bifidobacterium adolescentis*. Biotechnol Lett 21: 441-445

Van Laere KMJ, Schols HA, Voragen AGJ (1994) Tailor made produktie van oligosacchariden VMT 22: 33-35

Van Laere KMJ, Voragen CHL, Kroef T, Van den Broek LAM, Beldman G, Voragen AGJ (1999). Purification and mode of action of two different arabinoxylan arabinfuranohydrolases from *Bifidobacterium adolescentis* DSM 20083. Appl Microbiol Biotechnol 51: 606-613

Van Laere KMJ, Hartemink R, Beldman G, Pitson S, Dijkema C, Schols HA, Voragen AGJ (1999) Transglycosidase activity of *Bifidobacterium adolescentis* DSM 20083 alpha-galactosidase. Appl Microbiol Biotechnol 52: 681-688

Van Laere KMJ, Abee T, Schols HA, Beldman G, Voragen AGJ (2000) Characterisation of a novel ß-galactosidase from *Bifidobacterium adolescentis* DSM 20083 active towards transgalactooligosaccharides. Appl Envir Microbiol: 66: 1379-1384

Van Laere KMJ, Hartemink R, Bosveld M, Schols HA, Voragen AGJ (2000) Fermentation of plant cell wall derived polysaccharides and their corresponding oligosaccharides by intestinal bacteria. J Agric Food Sci: In Press

Voragen AGJ, Pilnik W (1995) Pectins. In food polysaccharides and their applications ed AM Stephen, New York

## SUMMARY

The research described in this thesis was part of a multi-disciplinary research program at the Wageningen University on the role of non-digestible oligosaccharides (NDOs) in food and feed. Four departments were involved in the project: Human Nutrition and Epidemiology, Animal Nutrition, Food Microbiology and Food Chemistry. The aim of the research described in this thesis was to investigate the influences of the chemical structure of NDOs on their fermentation by intestinal bacteria and study the glycosylhydrolases involved in the degradation of NDOs.

To establish this, initially structurally different oligosaccharides were produced, using various production methods and raw materials. Secondly the fermentative degradation of the NDOs by various intestinal bacteria was studied and finally the glycosylhydrolases involved in the degradation of the NDOs were purified en characterised.

In chapter 2 new types of NDOs obtained from plant cell wall polysaccharides are described. Oligosaccharides were produced from soy arabinogalactan, sugar beet arabinan, wheat flour arabinoxylan, polygalacturonan, and a rhamnogalacturonan fraction from apple. This resulted in the production of arabino-, (arabino-)galacto-, (arabino-)xylo-, galacturono-, and rhamnogalacturono- oligosaccharides. The different NDOs were utilised completely after 48h by the intestinal bacteria using an *in vitro* fermentation-model. To obtain more information, on which strains possess the capability to utilise these NDOs, the fermentative degradation by relevant individual species of intestinal bacteria was studied *in vitro*. All of the tested substrates were fermented to some extent by one or more of the individual species of bacteria tested. *Bacteroides* spp. were able to utilise plant cell wall derived oligosaccharides in addition to their reported activity towards plant polysaccharides. *Bifidobacterium* spp. were able to utilise the rather complex plant cell wall derived oligosaccharides. *Clostridium* spp., *Klebsiella* spp. and *Escherichia coli* fermented some of the selected substrates. These studies give valuable information on the fermentative capability of the tested intestinal strains, but do not allow prediction of the fermentation *in vivo*. *Bifidobacterium adolescentis* could utilise a wide range of NDOs and the utilisation of these substrates by the bacteria must be mediated by the glycosylhydrolases they produce. As *Bi. adolescentis* is recognised as one of the major *Bifidobacterium* spp. of the adult intestinal microflora, the glycosylhydrolases involved in the degradation of NDOs of the type strain of *Bi. adolescentis* were further studied.

In chapter 3 the purification of an arabinoxylan arabinofuranohydrolase (AXH-d3) from a cell-free extract of *Bi. adolescentis* DSM 20083 is described. Using an arabinoxylan derived oligosaccharide containing double-substituted xylopyranosyl residues, it was established that the enzyme specifically released terminal arabinofuranosyl residues linked to C-3 of double-substituted xylopyranosyl residues. For the complete degradation of the arabinoxylooligosaccharides by *Bi. adolescentis,* a ß-xylosidase and another arabinofuranohydrolase (AXH-m23) were involved. In Chapter 4 the purification of this other AXH-m23 from *Bi. adolescentis* is described and the mode of action of both new

arabinofuranohydrolases was studied in more detail. The production of both enzymes was induced upon growth of *Bi. adolescentis* on xylose and arabinoxylan-derived oligosaccharides. They were only active towards arabinoxylans and therefore denoted as arabinoxylan arabinofuranohydrolases. AXH-m23 released only arabinosyl groups which were linked to the C-2 or C-3 position of single-substituted xylose residues in arabinoxylan oligomers. AXH-d3 hydrolysed C-3 linked arabinofuranosyl residues of double-substituted xylopyranosyl residues of arabinoxylans and arabinoxylan-derived oligosaccharides. No activity was observed towards C-2 linked arabinofuranosyl residues of double-substituted xylopyranosyl residues, or against C-2 and C-3 linked arabinofuranosyl residues of single-substituted xylopyranosyl residues. Arabinoxylan from wheat flour was debranched almost completely by AXH-d3 in combination with AXH-m from *Aspergillus awamori*. The arabinoxylan arabinofuranohydrolases from *Bi. adolescentis* showed no activity towards *p*-nitrophenyl-α-L-arabinofuranoside or towards sugar beet arabinan, soy arabinogalactan, arabinooligosaccharides and arabinogalactooligosaccharides. In addition to the two arabinose releasing enzymes described in this thesis, other arabinofuranosidase(s), active towards the arabino- and arabinogalactooligosaccharides, are produced by *Bi. adolescentis*. This shows that *Bi. adolescentis* produces various specific enzymes which are active towards plant cell wall derived substrates, and only by using specific substrates these enzymes can be discovered.

Bifidobacteria are also known to have a high α-galactosidase activity, which enables them to utilise the NDOs raffinose and stachyose. This enzyme was purified from the cell-free extract of *Bi. adolescentis* DSM 20083. The α-galactosidase was found to act with retention of configuration (α→α), releasing α-galactose from *p*-nitrophenyl galactoside. This hydrolysis probably operates with a double displacement mechanism, and is consistent with the observed transglycosylation activity used for the production of new types of α-galactosides. Starting from melibiose, raffinose and stachyose, oligosaccharides could be formed. The oligosaccharides formed from melibiose could be identified as α-D-Gal*p*- (1→6)- α-D-Gal*p*-(1→6)-D-Glc*p* and α-D-Gal*p*- (1→6)- α-D-Gal*p*-(1→6)- α-D-Gal*p*-(1→6)-D-Glc*p*. This indicated that the transgalactosylation to melibiose occurred selectively at the C-6 hydroxyl group of the galactosyl residue. The trisaccharide α-D-Gal*p*- (1→6)- α-D-Gal*p*- (1→6)-D-Glc*p* formed could be utilised by various intestinal bacteria, including various bifidobacteria, and might therefore be an interesting pre- and synbiotic substrate.

*Bi. adolescentis* also produces a wide range of glycosidases upon growth on transgalactooligosaccharides (TOS) among them a novel ß-galactosidase. In chapter 6 the effect of both reducing and non-reducing transgalactooligosaccharides on growth of *Bi. adolescentis* DSM 20083 and on the production of this novel ß-galactosidase (β-gal II) is described. In cells grown on TOS, in addition to the lactose degrading ß-galactosidase (ß-gal I), another ß-galactosidase (ß-gal II) was detected which showed activity towards TOS but not towards lactose. ß-gal II activity was at least 20-fold higher in TOS grown cells than when cells were grown on galactose, glucose or lactose. It was speculated that ß-gal II was located

membrane or cell wall associated. ß-gal II has an optimal activity at pH 6 and was not active below pH 5. Its optimum temperature is 35°C. The enzyme showed highest $V_{max}$ towards galactooligosaccharides with low DP. This is in agreement with the observation that during fermentation of TOS the di- and trisaccharides were fermented first. β-Gal II was active towards ß-galactosyl residues being 1→4; 1→6; 1→3 and 1↔1 linked, signifying its role in the metabolism of galactooligosaccharides by *Bi. adolescentis*. Also upon growth on a arabinogalactan hydrolysate containing both oligomeric and polymeric fragments, regulation of the ß-galactosidase activity in the cell-extracts of *Bi. adolescentis* was observed. In chapter 7 the effect of AGOS and TOS on growth of bacteria in faecal suspensions is described. TOS and AGOS degrading strains belonging to the genera *Bifidobacterium* and *Bacteroides* could be isolated from faecal slurry after incubation of faecal inocula with these substrates. *Bi. adolescentis* and *Bacteroides vulgatus* were chosen as representatives for both genera to study the effect of TOS and AGOS on the regulation of the ß-galactosidase activity. The highest levels of ß-galactosidase were present in the cell-extract containing the cell-associated and intracellular enzymes. Both substrates regulated the cell-bound oligosaccharide degrading activity of *Bi. adolescentis* but not of *B. vulgatus*. The ß-galactooligosaccharide degrading activity of *B. vulgatus* was found to be present extracellularly. TOS and AGOS both increased the galactan degrading activity of *Bi. adolescentis*, whereas this increased activity towards polymeric galactan for *B. vulgatus* was only reached after growth on AGOS. These results suggest that growing *Bi. adolescentis* on TOS and AGOS results in the regulation of galactose-releasing enzymes which allow the bacteria to use both polymeric and oligomeric galactans.

Whether TOS also effected the glycosidase regulation *in vivo* was studied further. In chapter 8 of this thesis, results from two human trials aimed at establishing whether fructooligosaccharides and transgalactooligosaccharides would change the bacterial glycosidase activity in the colon are given. Transgalactooligosaccharides significantly increased the activity of the ß-galactosidase activity in the faecal water by 123%. Fructooligosaccharides did not have a significant regulatory effect on the ß-galactosidase production but significantly decreased α-L-arabinofuranosidase activity by 35%. Neither fructooligosaccharides nor transgalactooligosaccharides affected the activities of ß-glucosidase or ß-glucuronidase, which are enzymes involved in the generation of toxic compounds. It could be concluded from this study that specific regulation of ß-galactosidase activity occurs upon growth on transgalactooligosaccharides. Although the origin and specificity of the increased ß-galactosidases is not yet known, it can be concluded that glycosidase activity of the intestinal bacteria might be a useful biomarker of the colonic metabolic activity.

## SAMENVATTING

Oligosacchariden komen van nature voor in veel gewassen die worden gebruikt als grondstof bij de productie van voedingsmiddelen. Ze worden vaak, net als voedingsvezel, niet afgebroken worden door de spijsverteringsenzymen en worden daarom niet-verteerbare oligosacchariden (NDOs) genoemd. In de dikke darm kunnen ze dan vervolgens gefermenteerd worden door de darmbacteriën. Wanneer deze NDOs selectief de groei en/of activiteit van gunstige darmbacteriën, waaronder bifidobacteriën en lactobacillen, stimuleren worden gunstige gezondheidseffecten aan deze NDOs toegeschreven. Niet-verteerbare voedingscomponenten welke over deze eigenschappen beschikken worden prebiotica genoemd. Van diverse structureel verschillende oligosacchariden (waaronder fructooligosacchariden en transgalactooligosacchariden) wordt geclaimd dat ze prebiotisch werken.

Oligosacchariden zijn koolhydraten opgebouwd uit ongeveer drie tot tien suikereenheden. Ze verschillen onderling in de aanwezige bouwstenen, bindingstypes en de vertakkingsgraad. Ze kunnen geproduceerd worden door (enzymatische) hydrolyse van polysacchariden en door transglycosylering van disacchariden. Omdat vanuit verschillende bronnen nieuwe typen oligosacchariden gevormd kunnen worden, was een belangrijke vraagstelling van dit onderzoek in welke mate de chemische structuur de afbraak door darmbacteriën beïnvloedt en wat de invloed van deze NDOs is op de regulatie van de glycosidase activiteit van de darmbacteriën.

Om dit te onderzoeken werden in eerste instantie vanuit diverse grondstoffen structureel verschillende oligosacchariden geproduceerd. In hoofdstuk 2 wordt productie van oligosaccharide vanuit planten celwand polysacchariden beschreven. Arabino-, (arabino-)galacto-, (arabino-)xylo-, galacturono- en rhamnogalacturonooligosacchariden werden gevormd vanuit respectievelijk suikerbiet araban, soja arabinogalactaan, arabinoxylaan van tarwe bloem, polygalacturonaan en een rhamnogalacturonaan fractie van appel. Planten celwand polysacchariden zijn door hun diverse structuur een interessante grondstof voor de productie van oligosacchariden.

Oligosacchariden kunnen ook geproduceerd worden via transglycosylering reacties. In hoofdstuk 5 staat de productie van $\alpha$-galactooligosacchariden met een $\alpha$-galactosidase van *Bifidobacterium adolescentis* beschreven. $\alpha$-Galactooligosacchariden worden gefermenteerd door verschillende stammen behorend tot de bifidobacteriën en zijn daarom potentieel interessante prebiotica. Nieuwe types $\alpha$-galactooligosacchariden konden gevormd worden vanuit melibiose, De transgalactosylering van melibiose gebeurde selectief aan de C-6 hydroxylgroep van het galactose-eenheid. Transglycosylering reacties hebben het voordeel dat verschillend nieuwe bindingen gevormd kunnen worden, en dat verschillende donoren en acceptoren kunnen worden gebruikt. Een beperking is echter dat naast sucrose, maltose en lactose, weinig goedkope grondstoffen voorhanden zijn waardoor weinig variatie in bouwstenen mogelijk is.

Het tweede belangrijke doel van het onderzoek was om te bestuderen of structureel verschillende oligosacchariden selectief konden worden gefermenteerd door gunstige darmbacteriën. De verschillende typen oligosacchariden werden volledig afgebroken in een *in vitro* fermentatiemodel waarbij gebruik gemaakt werd van een fecale slurrie. Aangezien hierin meer dan 400 verschillende soorten darmbacteriën aanwezig zijn, was het niet duidelijk welke bacteriën de oligosacchariden konden afbreken. Om meer inzicht te verkrijgen werd onderzocht in welke mate oligosacchariden worden afgebroken door een selectie van darmbacteriën (hoofdstuk 2 en 5). Dit gaf ons de mogelijkheid om de fermentatie capaciteit van een aantal bacteriën in kaart te brengen. De oligosacchariden werden echter in het *in vitro* systeem niet alleen door bifidobacteriën gefermenteerd. Ook stammen behorende tot de Bacteroides konden niet alleen verschillende planten celwandpolysacchariden fermenteren maar ook de daaruit bereide oligosacchariden. De bifidobacteriën waren ook in staat de vaak complexe oligosacchariden afkomstig van plantencelwanden af te breken. Daartoe dienen ze enzymen te produceren welke de oligosacchariden afbreken tot mono- en disacchariden die verder benut kunnen worden door de darmbacteriën. *Bi. adolescentis* brak verschillende oligosacchariden volledig of gedeeltelijk af. Een van de meest voorkomende bifidobacteriën voorkomend in volwassenen is *Bi. adolescentis*. Een aantal glycosylhydrolases welke geproduceerd worden door *Bi. adolescentis* werde verder opgezuiverd en gekarakteriseerd.

In hoofdstuk 3 en 4 wordt de zuivering en karakterisering van 2 arabinoxylaan arabinofuranohydrolases die betrokken bij de afbraak van arabinoxylooligosacchariden beschreven. Door gebruik te maken van een model-substraat werd het werkingsmechanisme van de verschillende enzymen opgehelderd. AXH-d3 was in staat om arabinose eenheden van dubbel vertakte xylose eenheden te splitsen. Dit enzym is nog nooit eerder beschreven en zou toegepast kunnen worden bij de modificatie van verschillende arabinoxylanen in de levensmiddelenindustrie. Voor de volledige benutting van de arabinoxylooligosacchariden produceert *Bi. adolescentis* een tweede arabinoxylaan arabinofuranohydrolase welke in staat is arabinose eenheden te verwijderen van enkel vertakte xylose eenheden van het oligosaccharide. De productie van beide enzymen door *Bi. adolescentis* werd gestimuleerd door groei op xylose en arabinoxylooligosacchariden. Beide enzymen hadden geen activiteit op *p*-NP-arabinofuranoside, een synthetisch substraat en ook niet op andere arabinose houdende substraten zoals arabinogalactaan en arabanen en oligosacchariden welke daarvan afgeleid worden. Aangezien ze alleen actief waren op arabinoxylanen werden ze arabinoxylaan arabinofuranohydrolases genoemd.

In hoofdstuk 6 worden de ß-galactosidases beschreven welke betrokken zijn bij de afbraak van transgalactooligosacchariden (TOS) door *Bi. adolescentis*. Wanneer *Bi. adolescentis* gekweekt werd op TOS werd naast een lactose afbrekend ß-galactosidase (ß-gal I) een tweede ß-galactosidase (ß-gal II) gevormd. ß-gal II heeft wel activiteit op TOS maar is niet actief op lactose. De ß-gal II activiteit in de enzym-extracten was minimaal 20-keer hoger wanneer *Bi. adolescentis* gekweekt werd op TOS in plaats van op lactose, galactose of glucose. ß-gal II was niet extracellulair aanwezig en er zijn aanwijzingen dat het enzym cel-gebonden is. Het

enzym kan galactose afsplitsen van ß-1→4, ß-1→6; ß-1→3 en 1↔1 gebonden galactose eenheden en wordt als belangrijk beschouwd in het metaboliseren van TOS. Regulatie van de ß-galactosidase activiteit in de enzym-extracten van *Bi. adolescentis* werd ook waargenomen wanneer *Bi. adolescentis* gekweekt werd op een hydrolysaat van soja arabinogalactaan (AGOS) welke zowel oligomeer als polymeer galactaan bevat.

In hoofdstuk 7 worden de effecten van TOS en AGOS op de groei van bacteriën in een fecale suspensie beschreven. TOS en AGOS afbrekende bifidobacteriën en Bacteroides bacteriën konden geïsoleerd uit de faecal slurrie. *Bi. adolescentis* en *Bacteroides vulgatus* werden gekozen als model-organisme voor de beide genera. Groei op AGOS resulteerde in een toename van de ß-galactosidase activiteit in de enzym-extracten van *B. vulgatus*. TOS en AGOS stimuleerden beiden de ß-galactosidase en galactaan afbrekende activiteit van *Bi. adolescentis*. Dit suggereert dat na groei van *Bi. adolescentis* op TOS of AGOS de bacterie ook in staat is het polymeer galactaan af te breken.

In hoofdstuk 8 werden de gegevens gecombineerd verkregen uit twee voedingsproeven met gezonde vrijwilligers. Beide studies waren placebo gecontroleerde interventie studies waar de effecten van respectievelijk 15 gram fructooligosacchariden (FOS) en 15 gram TOS op de glycosidase activiteit in fecaal water bestudeerd werden. TOS verhoogde de activiteit van ß-galactosidase met 123%. FOS, daarentegen had geen significante effecten op de ß-galactosidase activiteit maar verlaagde de activiteit van arabinofuranosidase met 35%. De conclusie die hieruit getrokken kan worden is dat een specifieke regulatie van ß-galactosidase activiteit optreedt wanneer TOS als niet-verteerbare oligosacchariden worden gebruikt. De glycosidase activiteit zou een interessante biomarker kunnen zijn voor metabole activiteit in de dikke darm.

## CURRICULUM VITAE

Katrien Van Laere werd geboren op 4 mei 1969 in Deinze (België) Na het behalen van haar Gymnasium diploma in 1988 aan het Katholieke Gymnasium St-Bavo in Gent (België) begon zij in oktober van dat zelfde jaar met de studie Landbouwkundig ingenieur aan de Landbouw Universiteit Gent. Na het behalen van haar kandidaats-examen vervolgde zij haar studie met als hoofdrichting Humane Voeding. Haar afstudeervak voerde zij uit bij Levensmiddelenchemie (prof. dr. ir Hyghebaert) waar zij onderzoek deed aan de retrogradatie van zetmeel. Na het behalen van haar universitaire bul in september 1992, werkte ze aan pectine afbrekende enzymen aan de Landbouwuniversiteit Wageningen bij de vakgroep Levensmiddelenchemie. Van september 1993 tot April 1998 werkte zij als assistent in opleiding bij de sectie Levensmiddelenchemie en –microbiologie van de landbouwuniversiteit Wageningen. Het onderzoek uitgevoerd in deze periode staat beschreven in dit proefschrift, en werd begeleid door prof. dr. ir. AGJ Voragen, dr. G Beldman en dr. ing. HA Schols. Tijdens de AIO-periode werd haar zoon David geboren, en in de aansluitende periode waarin het proefschrift werd afgerond, werd haar tweede zoon Arnaud geboren. Sinds september 1997 is ze als onderzoeker in dienst bij Numico Research.

# List of publication

*Full papers*

Alles MS, Hautvast JG, Nagengast FM, Hartemink R, Van Laere KMJ, Jansen JB 1996 **Fate of fructo-oligosaccharides in the human intestine**. Br J Nutr 76: 211-221

Beldman G, Broek LAM van den, Schols HA, Searle Van Leeuwen MJF, Van Laere KMJ, Voragen AGJ (1996) **An exogalacturonase from** *Aspergillus aculeatus* **able to degrade xylogalacturonan**. Biotechnol Lett 18: 707-712.

Hartemink R, Van Laere KMJ, Mertens AKC and Rombouts FM (1996) **Fermentation of xyloglucan by intestinal bacteria** Anaerobe 2: 223-230

Alles MS, Katan MB, Salemans JM, Van Laere KMJ, Gerichhausen MJ, Rozendaal MJ, Nagengast FM (1997) **Bacterial fermentation of fructooligosaccharides and resistant starch in patients with an ileal pouch-anal anastomosis**. Am J Clin Nutr 66: 1286-1292

Van Laere KMJ, Beldman G, Voragen AGJ (1997) **A new arabinofuranohydrolase from** *Bifidobacterium adolescentis* **able to remove arabinosyl residues form double-substituted xylose units in arabinoxylan**. Appl Microbiol Biotechnol 47: 231-235

Hartemink R, Van Laere KMJ, Peeters CCKM, Nout MJR, Rombouts FM (1997) *In vitro* **cariogenicity of transgalactosyl oligosaccharides**. Lett Appl Bact 25: 38-42

Hartemink R, Van Laere KMJ, Rombouts FM (1997) **Growth of enterobacteria on fructooligosaccharides**. J Appl Micr 83: 367-374

Fransen CTM, Van Laere KMJ, Wijk AAC van, Brull LP, Dignum M, Thomas Oates JE, Haverkamp J, Schols HA, Voragen AGJ, Kamerling JP, Vliegenthart JFG (1998) **Alpha-D-Glcp-(1 α 1)-beta-Galp-containing oligosaccharides, novel products from lactose by the action of beta-galactosidase**. Carbohydr Res 314: 101-114

Alles MS, Hartemink R, Meyboom S, Harryvan JL, Van Laere KMJ, Nagengast FM, Hautvast JGAJ, (1999) **Effect of transgalactooligosaccharides on the composition of the human intestinal microflora and on putative risk markers for colon cancer**. Am J Clin Nutr 69: 980-991

Van Laere KMJ, Voragen CHL, Kroef T, Van den Broek LAM, Beldman G, Voragen AGJ (1999) **Purification and mode of action of two different arabinoxylan arabinofuranohydrolases from** *Bifidobacterium adolescentis* **DSM 20083** Appl Microbiol Biotechnol 51: 606-613

*List of Publications*

Van Laere KMJ, Hartemink R, Beldman G, Pitson S, Dijkema C, Schols HA, Voragen AGJ (1999) **Transglycosidase activity of** *Bifidobacterium adolescentis* **DSM 20083 alpha-galactosidase** Appl Microbiol Biotechnol. 52: 681-688

Van den Broek LAM, Ton J, Verdoes JC, Van Laere KMJ, Voragen AGJ, Beldman G (1999) **Synthesis of alpha-galacto-oligosaccharides by a cloned alpha-galactosidase from** *Bifidobacterium adolescentis*. Biotechnol Lett 21: 441-445

Van Laere KMJ, Abee T, Schols HA, Beldman G, Voragen AGJ (2000) **Characterisation of a novel ß-galactosidase from** *Bifidobacterium adolescentis* **DSM 20083 active towards transgalactooligosaccharides.** Appl Envir Microbiol 66: 1379-1384

Van Laere KMJ, Hartemink R, Bosveld M, Schols HA, Voragen AGJ (2000) **Fermentation of plant cell wall derived polysaccharides and their corresponding oligosaccharides by intestinal bacteria.** J Agric Food Sci: In Press

*Other papers*

Schols HA, Van Laere KMJ, Voragen AGJ (1994) **Determination of oligosaccharides.** Cost 92, metabolic and physiological aspects of dietary fibre in food, Copenhagen (DK), 1994, Recent progress in the analysis of dietary fibre, European Commision, Luxembourg, 1995, 53-59

Van Laere KMJ, Schols HA, Voragen AGJ (1994) **Tailor-made production of oligosaccharides.** VMT 27:33-35

Van Laere KMJ (1996) **Kleine, onverteerbare koolhydraten stimuleren de darmflora** Chemisch Magazine 385-388

Van Laere KMJ, Schols HA, Beldman G, AGJ Voragen (1997) **The influence of the chemical structure of oligosaccharides on the fermentability by intestinal bacteria** Med Fac Landbouww Univ Gent 62/4a

Houdijk JGN, Hartemink R, Van Laere KMJ, Bosch MW, Tamminga S, Verstegen MWA (1997) **Fructooligosaccharides and transgalactooligosaccharides in weaner pigs'diet.** In: Proceeding of the symposium 'Non-digestible oligosaccharides: healthy food for the colon?' hartemink R (Compiler): pp 69-79 Drukkerij Modern BV, Bennekom, The Netherlands

Van Laere KMJ, Bosveld M, Schols HA, Beldman G, Voragen AGJ (1997) **Fermentative degradation of plant cell wall derived oligosaccharides by intestinal bacteria.** In: Proceeding of the symposium 'Non-digestible oligosaccharides: healthy food for the colon?' Hartemink R (Compiler): pp 37-46 Drukkerij Modern BV, Bennekom, The Netherlands