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In vitro fermentation of selected xylo-oligosaccharides by piglet intestinal microbiota

Patrícia Moura [a,b], Susana Cabanas [a], Paula Lourenço [a], Francisco Gírio [a], Maria C. Loureiro-Dias [c], M. Paula Esteves [a,*]

[a] *Instituto Nacional de Engenharia, Tecnologia e Inovação, Departamento de Biotecnologia, Estrada do Paço do Lumiar, 22, 1649-038 Lisboa, Portugal*
[b] *Egas Moniz, Cooperativa de Ensino Superior, Quinta da Granja, 2829-511 Caparica, Portugal*
[c] *Instituto Superior de Agronomia, Universidade Técnica de Lisboa, 1349-017 Lisboa, Portugal*

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Abstract

The objective of this study was to compare the *in vitro* fermentability of xylo-oligosaccharides (XOS) with different degrees of polymerisation (DP) by the intestinal digesta collected in three distinct intestinal sections of the porcine intestinal tract: ileum, caecum, and distal colon. The studied oligosaccharides included commercial short-chain XOS (DP 2–5), and medium-chain (DP 2–14) and long-chain (DP 2–25) XOS obtained by autohydrolysis of brewery's spent grain (BSG), corn cobs (CC) and *Eucalyptus globulus* wood (EUC). The oligosaccharide and monosaccharide consumption, lactate and short-chain fatty acids concentrations were correlated with shifts on PCR titres of *Bacteroides/Prevotella*, *Bifidobacterium* and *Lactobacillus/Pediococcus* populations, by using group- and genus-specific primers.

All tested XOS were extensively fermented by the piglet ileal, caecal and colonic microbiota. The rate of consumption of medium- and long-chain XOS was notably reduced in the fermentations by the ileal inoculum as compared to commercial XOS. EUC XOS, CC XOS and commercial XOS supported an enhancement of bifidobacteria and lactobacilli replication in a first stage of the fermentations. Apparently this stimulation was not selective, because *Bacteroides/Prevotella* replication increased in a second stage of the fermentations, coincident in time with the highest consumption rates of some XOS mixtures tested. Mostly due to the slow fermentability by the ileal microbiota, medium-chain and long-chain XOS mixtures can be regarded as promising functional candidates suitable to act as distally fermentable substrates.

Keywords: Xylo-oligosaccharides; Piglet intestinal microbiota; Autohydrolysis; Degree of polymerisation; Prebiotic

1. Introduction

The prebiotic properties of some non-digestible oligosaccharides (NDO) and polysaccharides, namely fructo-oligosaccharides (FOS), transgalacto-oligosaccharides (TGOS) and inulin, have been extensively studied and there is great evidence of the physiological effects and health consequences of their consumption (Gibson, McCartney, & Rastall, 2005; Pool-Zobel, 2005; Watzl, Girrbach, & Roller, 2006). Other oligosaccharides (OS), such as xylo-oligosaccharides (XOS), isomalto-oligosaccharides (IMOS) and soy-oligosaccharides (SOS) are classified as ''emerging prebiotics'', presenting a promising prebiotic potential although they still lack of strong scientific evidence (Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004; Tuohy, Rouzaud, Bruck, & Gibson, 2005).

Okazaki, Fujikawa, and Matsumoto (1990) were pioneers in the study of the prebiotic effects of XOS. These authors observed a significant but transient increase in bifidobacterial numbers and organic acids levels in the faeces of human volunteers consuming short-chain XOS. Similar bifidogenic effects were described in rats and mice consuming XOS, more notably in animals presenting lower initial bifidobacterial numbers (Campbell, Fahey, & Wolf, 1997; Morishita, Yamada, Shiiba, Kimura, & Taniguchi, 1993). XOS-dependent bifidobacterial up-shifts observed *in vivo* are consistently supported

* Corresponding author. Tel.: +351 210924600; fax: +351 217163636.
E-mail address: paula.esteves@ineti.pt (M.P. Esteves).

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by *in vitro* studies (Crittenden et al., 2002; Jaskari et al., 1998; Moura, Barata, et al., 2007; Palframan, Gibson, & Rastall, 2003). However, the evidence for a selective stimulation of bifidobacterial growth by XOS has not been demonstrated. Microorganisms belonging to *Bacteroides*, *Clostridium* and *Lactobacillus* genera are also able to metabolise XOS mixtures mainly composed by xylobiose and xylotriose (Crittenden et al., 2002; Van Laere, Hartemink, Bosveld, Schols, & Voragen, 2000). However, more complex XOS structures have presented a higher capability to be selectivity fermented by bifidobacteria (Van Laere et al., 2000).

XOS can be produced by autohydrolysis of xylan-rich materials, such as byproducts from agricultural, agro-industrial and forestry origin (Garrote, Domínguez, & Parajó, 1999a; Garrote, Domínguez, & Parajó, 1999b). The process promotes hydrolysis of the hemicellulosic fraction of lignocellulosic materials and, although it consists of a non-selective fractionation process, the operational conditions can be optimised for XOS production. Chemically, XOS produced by autohydrolysis are constituted by a β-(1 → 4)-D-xylopyranose backbone which can be more or less substituted, depending on the feedstock material and the severity of the hydrothermal treatment (Carvalheiro, Garrote, Parajó, Pereira, & Gírio et al., 2005; Ebringerová, Hromádková, & Heinze, 2005). The crude XOS hydrolysates can be separated by gel filtration chromatography (GFC), and selected fractions can be pooled to obtain a desired average molecular weight range. This possibility enables the preparation of XOS mixtures with increased molecular weight, theoretically more capable to persist throughout the gastrointestinal (GI) tract and to support a saccharolytic fermentation up to more distal compartments of the intestinal tract when compared to short-chain XOS (Rastall & Maitin, 2002; van de Wiele, Boon, Possemiers, Jacobs, & Verstraete, 2007).

As NDO with promising prebiotic potential, XOS produced by autohydrolysis may constitute a dietary alternative to antimicrobial growth promoters, whose inclusion in animal diets has recently been banned in Europe (Patterson, 2005). In this study, we conducted a comparative study on the fermentability of commercial short-chain XOS and longer-chain XOS obtained by autohydrolysis. Our aim was to identify differences which could be of relevance concerning XOS prebiotic potential and its use as feed supplements.

2. Material and methods

2.1. Feedstock materials and preparation of purified XOS series

Chips of *E. globulus* wood (EUC) were obtained from ENCE Complejo Industrial de Pontevedra, Puentemolinos, Lourizan (Spain). Corn cobs (CC) and brewery's spent grain (BSG) were supplied by Casa Agrícola Monte Real, Salvaterra de Magos (Portugal) and Sociedade Central de Cervejas, Vialonga (Portugal), respectively. Crude XOS-rich hydrolysates of CC, obtained as previously described (Garrote et al., 1999b) were received from J.C. Parajó (University of Vigo, Ourense, Spain). Crude de-starched XOS-rich hydrolysates of BSG were obtained from F. Carvalheiro (Carvalheiro, Esteves, Parajó, Pereira, & Gírio, 2004). Gram-quantities of purified XOS series (DP 2−25) from EUC crude hydrolysates, prepared as described by Kabel, Schols, and Voragen (2002), were received from H. Schols (Wageningen University, Wageningen, the Netherlands).

CC and BSG XOS-rich hydrolysates were purified and characterized as described by Moura, Barata, et al. (2007). Briefly, the liquid phases obtained from the autohydrolysis treatments were pooled together for separation of xylo-oligosaccharides (XOS) in a preparative gel filtration chromatography (GFC) column BPG 100/950 (Amersham Pharmacia Biotech, Uppsala, Sweden) filled with Superdex 30™. The system was equipped with a refractive index detector (K-2401 Knauer, Berlin, Germany). Separated fractions were collected into plastic bottles using a Super-frac™ collector (Amersham Pharmacia Biotech) and characterised in terms of apparent molar mass in a LaChrom HPLC system (Merck, Darmstadt, Germany) equipped with three in-series size exclusion chromatography columns BIOSEP-SEC (Phenomenex, Torrance, USA), S4000, S3000 and S2000, using 50 mM $NaNO_3$ as eluent at 30 °C, and a RI detector (Merck L-7490). Calibration was performed with external standards of xylose, maltose, maltotriose, stachyose and dextrans (Sigma, St. Louis, USA) with molecular weights ranging from 1000 to 580,000 Da. Calibration was used to delimit the ranges of the GFC fractions of CC XOS and BSG XOS to be pooled together. The BSG XOS and CC XOS pools had an estimated apparent molar mass ranging from 1760 to 429 Da and 2160 to 286 Da, respectively, that correspond to average degrees of polymerisation (DP) from 12 to 3, and 14 to 2, respectively. The pools were freeze-dried (Labconco, Kansas City, USA) prior to the use in the fermentation assays. The Kjeldahl method was used to determine the nitrogen content of BSG XOS and the conversion factor of 5.83 (*AOAC Official Methods of Analysis, 1975*; FAO, 2002) was used to express the equivalent amount of protein.

2.2. Mono- and oligosaccharide composition of the XOS mixtures

The purified XOS series obtained from EUC, CC and BSG autohydrolysis and commercial XOS (Suntory Ltd., Osaka, Japan) were analysed by HPLC for quantitative carbohydrate analysis. The HPLC system (Waters, Milford, USA) was equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, USA) in combination with a cation H^+-guard column (Bio-Rad) and a refractive index detector (Waters 2410). Elution took place at 50 °C with 5 mM H_2SO_4. OS were quantified by quantitative acid hydrolysis (QAH) of the selected XOS series with 40 g/kg of H_2SO_4 at 121 °C for 60 min. OS concentrations were expressed as the increase in sugar monomers after QAH.

2.3. Intestinal samples

Intestinal samples were obtained from one healthy 34-day-old Duroc × Landrace male piglet, fed with a commercial

weaning diet based on barley and soybean meal. The animal was sacrificed after a 16 h fasting period for removal of the entire GI tract. Samples from the luminal contents of the terminal ileum, caecum and distal colon were collected into sterile vials. All vials were kept refrigerated until arrival to the laboratory and preparation of the intestinal inocula for the fermentation assays did not exceed 2 h after collection.

2.4. Fermentation media

The nutrient base medium used in the fermentation experiments was modified from Jaskari et al. (1998) and prepared as described previously (Moura, Barata, et al., 2007) except that a 100 mM solution of sodium hydroxide was used as solvent. The medium was deoxygenated with nitrogen and carbon dioxide using a gassing manifold system and pH was adjusted to 6.8. Nine-milliliter aliquots were distributed into airtight anaerobic culture tubes (Bellco Biotechnology Inc., Vineland, USA), which were capped with butyl rubber stoppers and sealed with aluminium caps (Bellco Biotechnology Inc.) before autoclave sterilisation. Anaerobic stock solutions of Yeast Nitrogen Base (YNB) (Difco, Detroit, Michigan, USA) and XOS were prepared in airtight serum bottles and distributed into the culture tubes, to final concentrations of 5.0 g/L of freeze-dried powder.

2.5. Fermentation experiment

The inocula for the fermentation experiments were prepared as described by Moura, Barata, et al. (2007). Briefly, the intestinal contents were diluted in reduced physiological saline (RPS), pH 6.8 (Hartemink, 1999) before inoculation. The tubes with the fermentation medium were inoculated with 0.2 mL of each intestinal dilution, in duplicate (CC XOS) or triplicate (EUC, BSG and commercial XOS) for each combination of inocula/sampling time (0, 11.5, 30 and 72 h), and incubated at 37 °C for 72 h. At each sampling time, cells were harvested by centrifugation and supernatants were filtered for HPLC analysis.

2.6. DNA isolation and PCR

NucleoSpin Tissue Kit (Mackerey-Nagel, Düren, Germany) was used to isolate DNA of cells harvested from the fermentation experiments. Total DNA was serially diluted starting from 10 ng (correspondent to 1/1 dil.), to be used as template in PCR reactions. The primers used in this study are presented in Table 1. Amplification reactions with *Lactobacillus* group-specific primers were performed according to the optimized protocol previously described for FOS fermentations (Moura, Simões, Gírio, Loureiro-Dias, & Esteves, 2007). *Bifidobacterium* genus-specific primers were used according to Matsuki et al. (2002) and *Bacteroides/Prevotella* specific primers according to Menaia, Simões, Sousa, Moura, and Amaral-Collaço (1998). Electrophoresis was performed on 1% agarose gels in 1 × TAE buffer, and each lane was loaded with a fixed volume of sample. PCR products were visualised using ethidium bromide staining.

2.7. Quantification of carbohydrates and fermentation products in the fermentation supernatants

The filtered supernatants from the culture tubes were analysed for carbohydrates and main fermentation products (lactate, acetate, propionate and butyrate) by HPLC with an Aminex HPX-87H column (Bio-Rad, Richmond, USA) against external standards, as described above.

3. Results & discussion

3.1. Characterisation of the XOS mixtures

XOS produced by autohydrolysis derived from three different xylan-rich feedstock materials, namely *E. globulus* wood, corn cobs and brewery's spent grain. The total content in oligo- and monosaccharides of each XOS mixture is presented in Table 2. The composition of each XOS series is primarily determined by the xylan source used in the autohydrolysis treatments. Considering that some feedstocks used for autohydrolysis derived from arabinoxylan-rich materials, the amount of XOS in the mixtures was calculated considering the sum of xylose and arabinose equivalents released by QAH, and xylose-based OS were uniformly referred to as XOS. The glucose released after QAH possibly deriving from glucan of the feedstock materials was referred to as glucose oligomers (GlcOS).

The XOS selected for this study corresponded to mixtures with three different ranges of DP. Commercial XOS comprising mainly xylobiose and xylotriose can be classified as short-chain XOS. The XOS series up to DP 14 from corn cobs (CC XOS) and from brewery's spent grain (BSG XOS) can be referred to as medium-chain XOS. XOS mixtures from

Table 1
Genus- and group-specific primers used in this study

Targeted genus or group	Primer designation	Primer sequence (5′ to 3′)	Source or reference
Lactobacillus/Pediococcus	Lab0677F	CTCCATGTGTAGCGGTG	Heilig et al. (2002)
	Lact71R	TCAAAACTAAACAAAGTTTC	Moura, Simões, et al. (2007)
Bifidobacterium	Bifid-F	CTCCTGGAAACGGGTGG	Matsuki et al. (2002)
	Bifid-R	GGTGTTCTTCCCGATATCTACA	Matsuki et al. (2002)
Bacteroides/Prevotella	BactrF1	GGGGTTCTGAGAGGAAG	Menaia et al. (1998)
	BactrF2	ACCCCCCATTGTAACAC	Menaia et al. (1998)

Table 2
Characterisation of the XOS mixtures used in the fermentations by piglet intestinal inocula. Oligomeric carbohydrates were determined as monomeric equivalents released after QAH

	EUC XOS	CC XOS	BSG XOS	Commercial XOS
DP range	2–25	2–14	3–12	2–5
XOS[a]	807	709	411	833
GlcOS[a]	30	57	90	58
Glucose[a]	7	ND	ND	10
Xylose[a]	9	16	11	5
Arabinose[a]	ND	19	4	ND
Others[a]	147	199	484	94
Ara/Xyl[b]	0	2	20	11
AcO/Xyl[b]	43	20	12	1
UA/Xyl[b]	nd	nd	nd	nd

ND, not detected; nd, not determined; EUC XOS, xylo-oligosaccharides produced from *E. globulus* wood; CC XOS, xylo-oligosaccharides produced from corn cobs; BSG XOS, xylo-oligosaccharides produced from brewery's spent grain; DP, degree of polymerisation; GlcOS, glucose oligomers; Ara, arabinose; Xyl, Xylose; AcO, acetyl substituents; UA, uronic acids.

[a] g/kg dry weight.

[b] mol/100 mol − molar percentage of released arabinose or acetic acid per released xylose, after QAH.

Eucalyptus wood (EUC XOS) comprising molecules up to DP 25 can be designated as long-chain XOS. The IUB-IUPAC nomenclature defines oligosaccharides as saccharides containing between 2 and 10 sugar moieties (IUB-IUPAC, 1982), although molecules with a molecular range up to 19 monosaccharide units can be considered as NDO (Voragen, 1998). Under a nutritional point of view, oligomeric structures with DP up to 50 can be defined as resistant short-chain carbohydrates (RSCC), which cannot be hydrolysed by mammalian endogenous enzymes of the small intestine (Englyst & Englyst, 2005). According to this later definition, all XOS mixtures from Table 2 can be classified as RSCC and therefore constitute potential fermentation substrates at the hindgut level.

Because BSG is known by its high protein content (Santos, Jiménez, Bartolomé, Gómez-Cordovés, & del Nozal, 2003), the nitrogen content of BSG XOS was determined (48 g/kg), which corresponds to 278 g/kg (dry weight basis) when expressed as protein equivalents. Apparently, the fractionation step included in the present work was not efficient enough to purify BSG XOS (Table 2) and it can be speculated that hydrolysis products from BSG proteins were still included in the selected pool of BSG XOS. Among all the mixtures produced by autohydrolysis, only BSG XOS presented a considerable value for GlcOS (Table 2).

3.2. Consumption of OS and accumulation of lactate and SCFA in the fermentation media

The concentrations of XOS, free monosaccharides and SCFA in the fermentation media were monitored for 72 h (Fig. 1), so as GlcOS concentration of the BSG XOS mixture (Fig. 2). The amount of each OS mixture added to the culture media was standardised on the basis of dry weight of freeze-dried product (5 g/L), which introduced slight differences on mono- and oligosaccharides content in the culture media. The pH values did not decrease markedly during the 72 h of the fermentation trials (final pH $\geq$ 6.5).

In vitro experiments allow the comparison of rates to which OS are broken down and consumed in fermentation experiments. Therefore, these experiments are important to determine quantifiable parameters suitable for comparing the *in vitro* fermentability of the different substrates. The decline of XOS concentration in the growth media and the increase in organic acids throughout the fermentations were indicative of extensive microbial fermentation by all the inocula (Fig. 1). No accumulation of monosaccharides occurred as consequence of OS disappearance in the media (Fig. 1). The major differences on XOS consumption profiles occurred on XOS produced by autohydrolysis between the fermentations by the ileal and the large intestine inocula. In the fermentations by the ileal inoculum, the consumption profiles of medium- and long-chain XOS were similar throughout all fermentations. After 30 h, averagely 76% of the initial amount of these XOS remained in the media as fermentation substrate. Afterwards the concentrations slowly decreased up to 72 h. In the fermentations by the caecal or colonic inocula, medium- and long-chain XOS were all utilised to a similar extent (<15% residual) up to 30 h. The highest consumption rates by the caecal or colonic inocula of EUC XOS were determined between 11.5 and 30 h, whereas CC XOS and BSG XOS originated an almost constant consumption rate from 0 up to 30 h. Regarding the contribution of GlcOS of the BSG XOS mixture (Table 2), 84.2, 86.6 and 86.9% of the initial GlcOS were consumed by the microorganisms from, respectively, the ileal, caecal and colonic inocula up to 11.5 h. In opposition to autohydrolysis XOS, commercial XOS were rapidly consumed by the ileal microbiota, remaining in the medium only 25% of the initial amount after 30 h. Interestingly, the consumption pattern of commercial XOS by the inocula from the large intestine displayed a trend to resist an initial degradation up to 11.5 h resembled to that of EUC XOS. The slower fermentation rate of EUC XOS, CC XOS and BSG XOS by the ileal inoculum denotes a lower fermentation efficiency of the foregut microbiota towards longer molecules of XOS, when compared to short-chain XOS. In this study it was not possible to find a correlation between the rate of XOS consumption and their degree of substitution by arabinose or acetyl groups. In order to fulfil the prebiotic criteria (Gibson et al., 2004) XOS need to resist hydrolysis by the microbiota from the upper part of the piglet GI tract. Consequently, the slower fermentability of EUC XOS, CC XOS and BSG XOS by the ileal digesta may indicate their adequacy as more persistent OS, potentially capable of reaching more distal compartments of the porcine GI tract. In a previous study, a series of chemically diverse oligosaccharides including short-chain XOS were compared in their *in vitro* fermentability by porcine faecal digesta (Smiricky-Tjardes et al., 2003). The slow rate to which short-chain XOS were fermented led the authors to suggest that such XOS would constitute a suitable source of fermentable substrate for the distal portion of the pig GI tract. Our data showed that short-chain XOS were much more

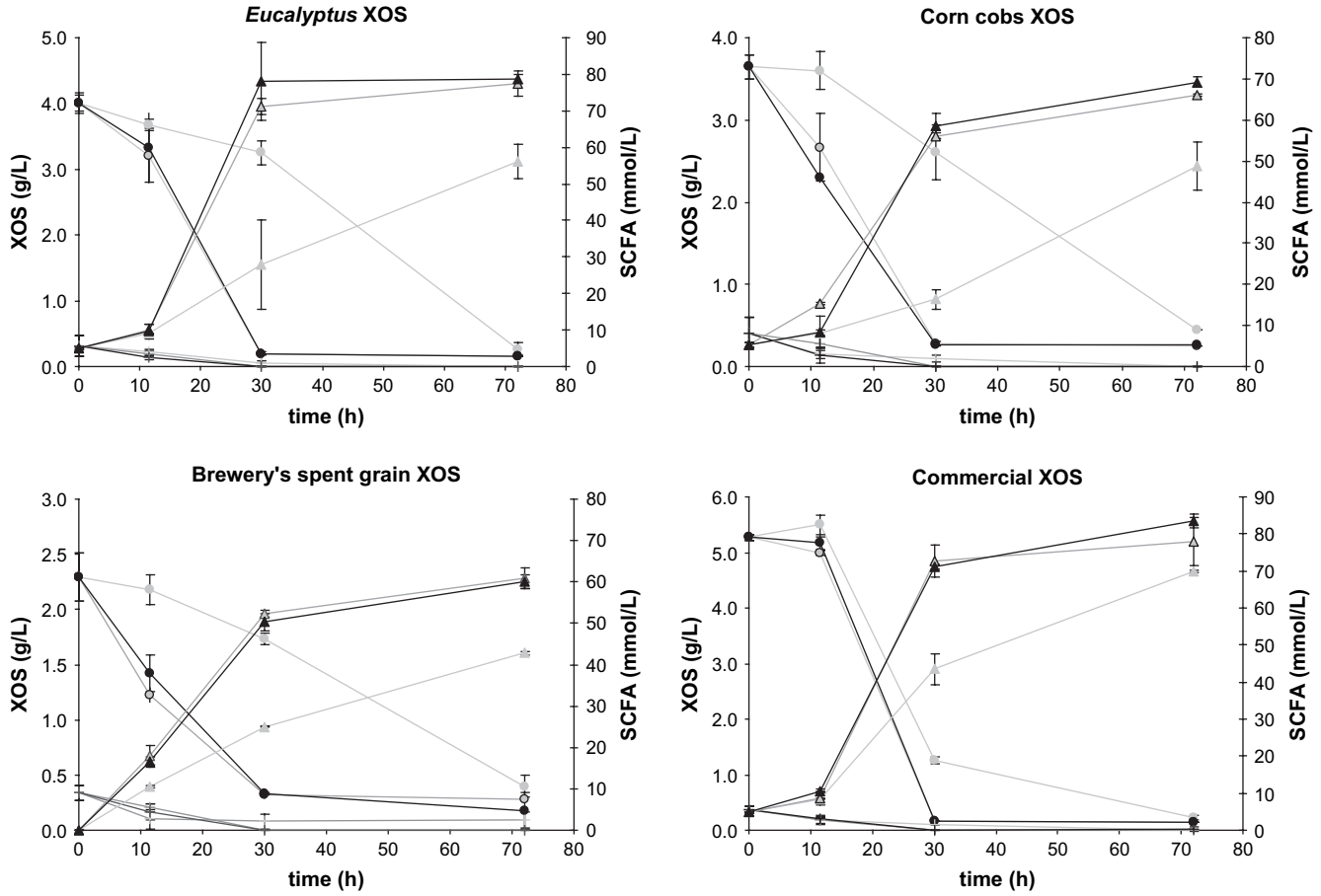

Fig. 1. Consumption of XOS and SCFA accumulation during 72 h of *in vitro* fermentation of XOS mixtures from *Eucalyptus* wood (EUC XOS), corn cobs (CC XOS), brewery's spent grain (BSG XOS) and commercial XOS by the ileal, caecal and colonic inocula. (○ XOS, △ SCFA, + free monosaccharides; ● Ileum; ◎ Caecum; ● Colon; ▲ Ileum; ◬ Caecum; ▲ Colon; + Ileum; + Caecum; + Colon).

rapidly consumed than EUC XOS, CC XOS or BSG XOS by the ileal digesta. Comparing the consumption profiles, it is possible to advocate an even greater capability of the latter XOS to persist throughout the porcine GI tract.

In vitro methods are also valuable to measure the production of organic acids since these are not absorbed or metabolized as if they were produced *in vivo*. By using samples from piglet digesta collected in different gut compartments, not only the extent of XOS fermentation, but also the type and relative amounts of organic acids produced can be compared. Major SCFA resulting from fermentation in mammalian intestinal tract are acetate, propionate and butyrate, whereas lactate is an intermediate metabolite from the fermentation process (Macfarlane & Macfarlane, 2003). The amounts of these organic acids accumulated during *in vitro* fermentation of all the XOS mixtures are represented in Fig. 3. The inocula from the caecum and colon fermented XOS faster (more rapid accumulation of SCFA) and to a greater extent (greatest SCFA accumulation) than the ileal inoculum (Fig. 3). Lactate was typically detected in low concentrations in a first stage of the fermentations. In the fermentations by the ileal inoculum, lactate was detected up to 30 h, although increases from 11.5 to 30 h were only registered for commercial XOS and CC XOS. Lactate production at the beginning of the experiments is in accordance with the fact that it is an intermediary product of carbohydrate fermentation which can be converted to acetate, propionate and butyrate by common intestinal bacteria (Belenguer et al., 2006; Cotta & Whitehead, 1998; Duncan, Louis, & Flint, 2004). Lactate production can be correlated with the involvement of lactic bacteria and bifidobacteria early in the fermentation since they are able to utilise XOS

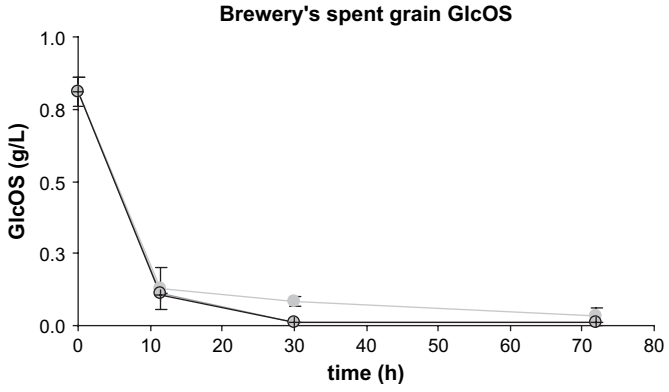

Fig. 2. Consumption of glucose oligomers (GlcOS) during 72 h of *in vitro* fermentation of XOS mixtures from brewery's spent grain (BSG XOS) by the ileal, caecal and colonic inocula. (● Ileum; ◎ Caecum; + Colon).

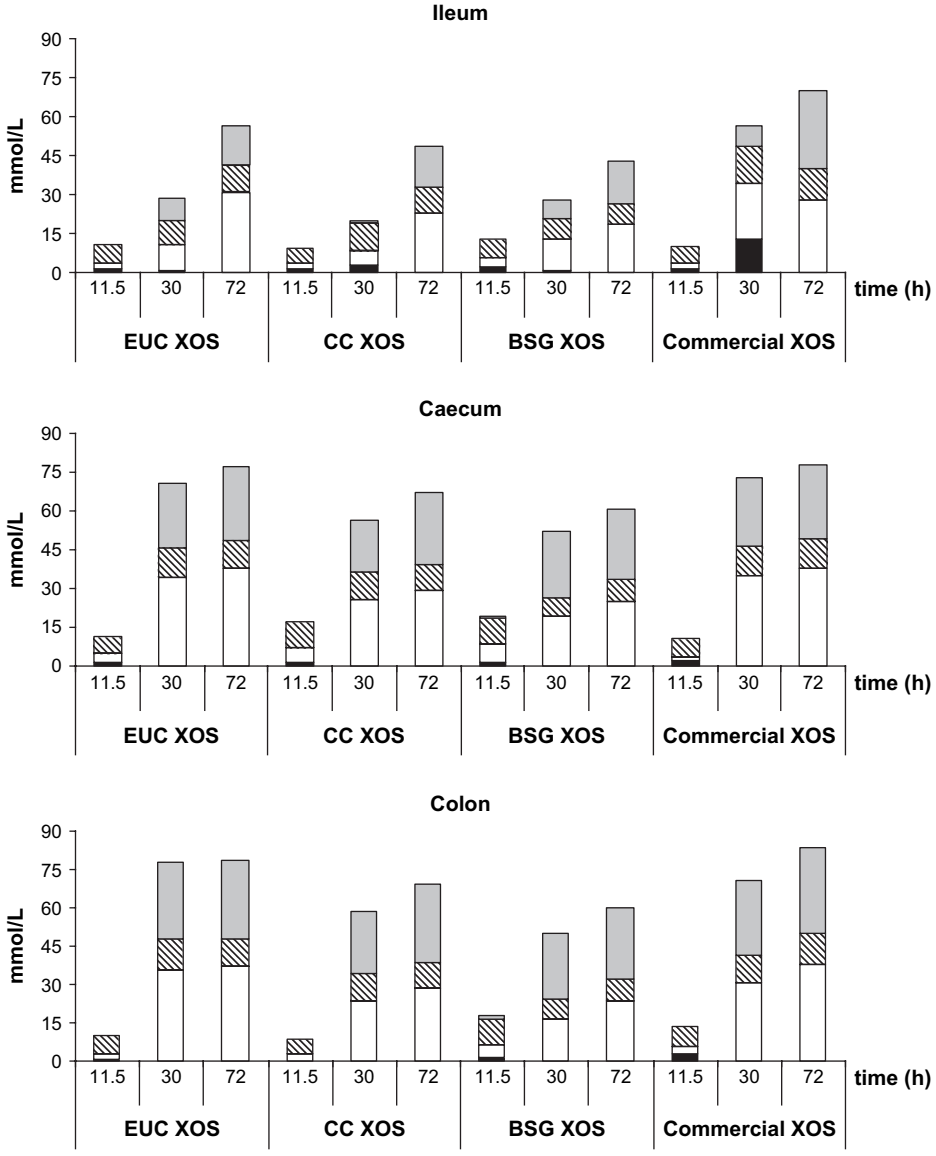

Fig. 3. Accumulation of SCFA and lactate during 72 h of *in vitro* fermentation of XOS mixtures from *Eucalyptus* wood (EUC XOS), corn cobs (CC XOS), brewery's spent grain (BSG XOS) and commercial XOS by the ileal, caecal and colonic inocula. (■ Lactate, □ Acetate, ▨ Propionate, ▩ Butyrate).

(Crittenden et al., 2002) producing lactate and acetate but not butyrate (Scardovi, 1988). Strains of *Selenomonas ruminantium* are also described as able to ferment XOS not only to lactate and acetate, but also producing propionate (Cotta & Whitehead, 1998). Members of this genus were reported to be present in the colonic wall and in the stomach of pigs (Mikkelsen, Højberg, & Jensen, 2007; Pryde, Richardson, Stewart, & Flint, 1999). In general, it was observed that propionate concentrations tended to stabilise up to 30 h or to decrease slightly afterwards. Acetate and butyrate were the major fermentation end-products in all cases; the former was detected in all sampling times, whereas butyrate only started to accumulate after 11.5 h. XOS acetogenic potential observed in this study is in agreement with previous data from *in vitro* fermentations of commercial XOS using pig faecal contents and *in vivo* experiments with rats fed with diets supplemented with commercial XOS (Imaizumi, Nakatsu, Sato, Sedarnawati, & Sugano, 1991; Smiricky-Tjardes et al., 2003). In the fermentations by the caecal and colonic inocula, the concentrations of both acetate and butyrate increased more rapidly, between 11.5 and 30 h. In the fermentations of EUC XOS and CC XOS by the ileal inoculum, acetate concentrations increased later, from 30 to 72 h, while butyrate concentrations suffered major increases from 30 to 72 h in all cases.

Exploring the differences between XOS mixtures, the concentrations of acetate and lactate after 30 h were considerably higher for commercial XOS than for any other XOS mixture in the fermentations by the ileal inoculum, suggesting a correlation with the rapid consumption of short-chain XOS observed from 11.5 to 30 h (Fig. 1). In a second stage of commercial XOS fermentation by the ileal digesta (>30 h), in which XOS were 25% residual in the fermentation media, butyrate accumulated up to a higher concentration as compared to all the other XOS. A better temporal correspondence was observed between

medium- and long-chain XOS consumption and butyrate production, suggesting the possibility of longer XOS to act as butyrogenic substrates. Butyrate arising from microbial fermentation is important for the energy metabolism and normal development of colonic epithelial cells and presents an important protective role in relation to colonic disease (Pryde, Duncan, Hold, Stewart, & Flint, 2002). However, the former assumption is in disagreement with results obtained in fermentations of medium-chain XOS by human faecal inocula, where butyrate was mainly produced when all XOS were already degraded (Kabel, Kortenoeven, Schols, & Voragen, 2002). Some authors refer that the butyrogenic effect of certain dietary substrates can be explained by lactate cross-feeding between genera capable of fermenting oligo- or polysaccharides, which are lactate-producers, and genera capable of utilising lactate, which are butyrate-producers (Belenguer et al., 2006; Duncan et al., 2004). Accordingly, considering that lactate reached a considerable concentration (13.0 mmol/L) in the fermentation of commercial XOS by the ileal inoculum during the first 30 h, it is expected that substantial lactate fermentation to butyrate has occurred. Since a rapid fermentation rate of lactate to butyrate and/or acetate may turn lactate undetectable, it is not possible to rule out the same hypothesis for the other fermentations of this study. Similar observations have already been registered in controlled co-cultures of lactate-producers and lactate-utilisers/butyrate-producers using starch as carbohydrate energy source (Duncan et al., 2004).

Considering the contribution of GlcOS in the mixture from BSG autohydrolysis and the fact that it was considerably consumed up to 11.5 h (Fig. 2), some differences could be expected in the fermentation products accumulated in the media during the same time period. In the fermentations by the colonic digesta, acetate and propionate concentrations were slightly higher at 11.5 h as compared to other XOS and very small amounts of butyrate were already detected up to 11.5 h in the fermentations by the caecal and colonic inocula.

3.3. PCR titres

The *Lactobacillus*/*Pediococcus* and *Bacteroides*/*Prevotella* group-specific and *Bifidobacterium* genus-specific primer pairs listed in Table 1 were used to determine the maximum dilution of total DNA able to produce PCR amplification (PCR titre). The PCR titres determined throughout XOS fermentations by the ileal, caecal and colonic inocula are presented in Fig. 4. Considering that the PCR titre corresponds to a detection limit (Wang, Cao, & Cerniglia, 1996) which depends on the proportion of targeted DNA among total DNA, it should allow to monitor shifts of targeted bacterial groups in mixed culture fermentations. The aim of determining PCR titres was to compare the efficacy of the different XOS mixtures to support the replication of bifidobacteria and lactobacilli, more specifically to evaluate XOS potential to act as selective substrate and/or to affect the targeted bacterial groups differently depending on the GI section from which each inocula was collected.

The PCR titres obtained with Lab0677F + Lact71R attained considerable values at the beginning of the fermentations. Except for BSG XOS, increases of the PCR titres with Lab0677F + Lact71R were only noticed from 0 up to 11.5 h for all the inocula. In the fermentations by the ileal inoculum, commercial XOS supported the major increase with the former primer pair. After 11.5 h, the PCR titres produced with Lab0677F + Lact71R and the ileal inoculum in the fermentations of commercial XOS decreased steadily up to 72 h. This decrease was less pronounced for CC XOS and the value of the PCR titre of EUC XOS at 30 h was maintained up to 72 h. In the fermentations by the caecal inoculum, increases in the PCR titres produced with Lab0677F + Lact71R were more pronounced for commercial XOS and EUC XOS. BSG XOS gave rise to the *Lactobacillus*/*Pediococcus* profile presenting the lowest increase from 0 up to 11.5 h in the fermentations by caecal inoculum. The maximal PCR titres from *Lactobacillus*/*Pediococcus* detection were attained up to 11.5 h in the fermentations of commercial XOS by the inoculum from the colon. Bifidobacteria were not detected in any sample at 0 h. However, increases in the PCR titres determined at 11.5 h in the fermentations of EUC XOS, CC XOS and commercial XOS by the ileal inoculum denoted that bifidobacterial replication was stimulated in a first stage of fermentation. BSG XOS were unable to raise notably bifidobacterial PCR titres in the fermentations by the ileal inoculum. In the fermentations by the caecal inoculum, the PCR titre profiles obtained with Bifid-F + Bifid-R showed an increase up to 11.5 h on EUC XOS and BSG XOS, and up to 30 h on CC XOS. Surprisingly, commercial XOS were unable to produce a notable increase on bifidobacterial detection limit in the fermentations by microorganisms from the caecum. The most consistent increases on *Bifidobacterium* PCR titres in the fermentations by the colonic inoculum were obtained for commercial XOS and CC XOS. In these cases, PCR titres increased from 0 to 11.5 h, more notably for commercial XOS than for CC XOS, and the values were maintained up to 30 h for CC XOS and up to 72 h for commercial XOS. According to the results of the PCR titres (Fig. 4), the replication of *Bifidobacterium* and *Lactobacillus*/*Pediococcus* was enhanced at the beginning of the fermentations, in most cases up to 11.5 h. Lactic acid bacteria are common inhabitants of the porcine GI tract (Jensen, 2001). *Lactobacillus*/*Enterococcus* constitute a stable population along the porcine intestinal tract (Franklin, Mathew, Vickers, & Clift, 2002; Mountzouris et al., 2006) which is in agreement with the PCR titres obtained with Lab0677F + Lact71R at the beginning of the fermentations. Irrespective of the inocula, the XOS with the highest impact (highest increase on the detection limit) on *Lactobacillus*/*Pediococcus* replication were commercial XOS. Particularly in the fermentations of commercial XOS by the ileal inoculum, increases on Lab0677F + Lact71R PCR titres were concomitant with the highest lactate concentrations up to 30 h (Fig. 3) which agrees with the proportional relevance of lactobacilli in the proximal intestinal regions of the porcine GI tract (Jensen, 2001). In opposition to lactic acid bacteria, bifidobacteria represent a minor proportion of the intestinal microbiota in the porcine GI tract (Mikkelsen, Bendixen, Jakobsen, & Jensen, 2003), which is in accordance with no amplification produced by PCR with the

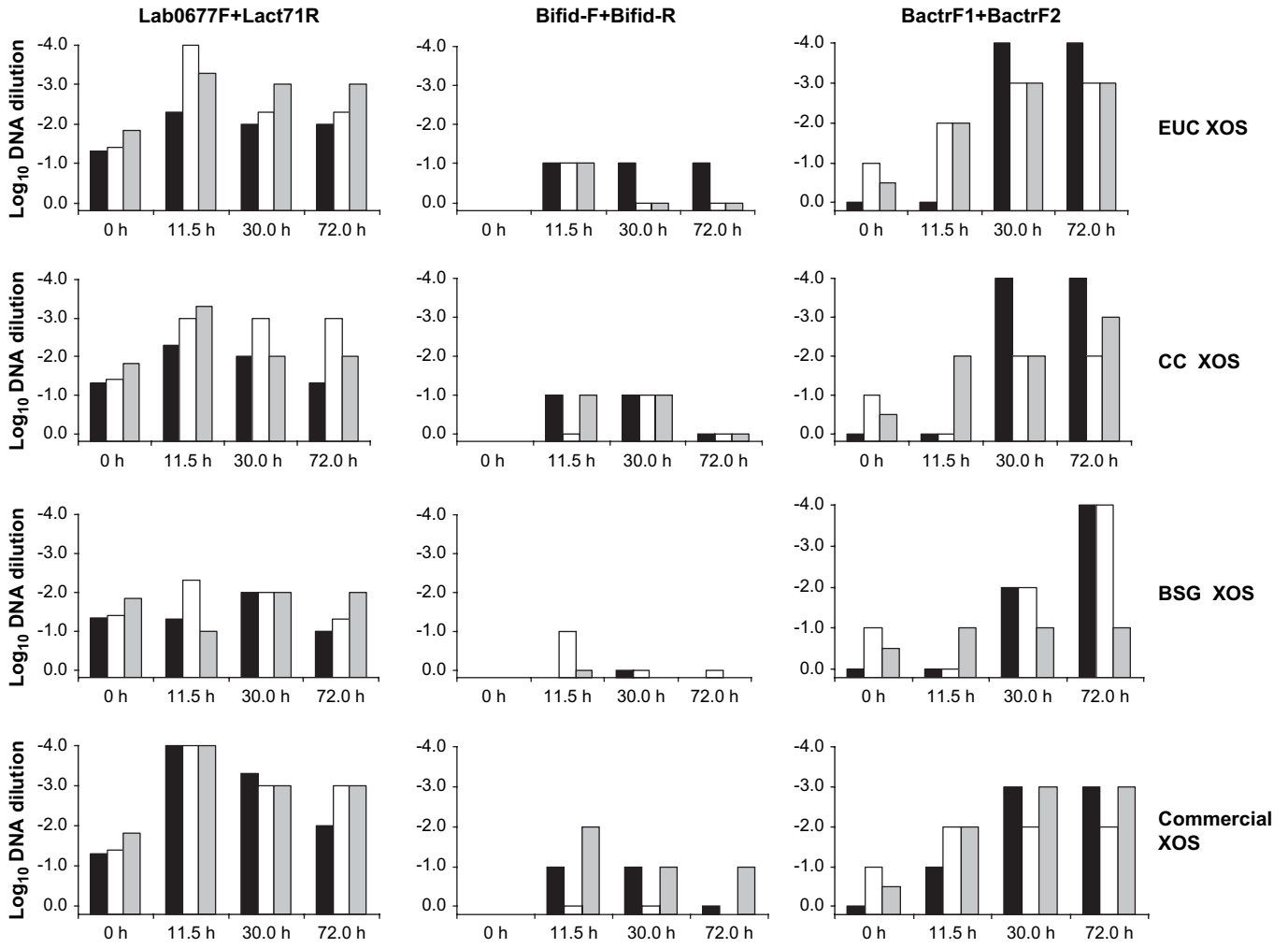

Fig. 4. Log_{10} values of the maximal dilutions of total DNA (isolated at 0, 11.5, 30 and 72 h from XOS fermentations by the ileal, caecal and colonic inocula) capable of producing the expected amplicon by PCR using the primer pairs Lab0677F + Lact71R, Bifid-F + Bifid-R or BactrF1 + BactrF2. Values were based in duplicates of independent positive PCR reactions, and values for 0 h were averaged for the respective inoculum and primer pair. (■ Ileum, □ Caecum, ▫ Colon).

primer pair Bif-F + Bif-R in the samples from 0 h. The subsequent increases on the PCR titres obtained with this primer pair indicate that bifidobacteria were able to replicate up to detectable levels in the first stage of most XOS fermentations. Commercial XOS demonstrated the highest efficacy (highest increase on the detection limit and subsequent maintenance of a detectable level of bifidobacteria) in stimulating bifidobacterial replication in the fermentation by the colonic inoculum, immediately followed by CC XOS. The preference of bifidobacteria to utilise short-chain XOS *in vitro* has already been described (Crittenden et al., 2002). *In vivo*, the bifidogenic character of short-chain XOS has been reported in humans and rats (Campbell, Fahey, & Wolf, 1997; Hsu, Liao, Chung, Hsieh, & Chan, 2004; Okazaki et al., 1990). This bifidogenic potential of XOS is a promising characteristic in pig nutrition since it is general consensus that the effect is inversely related to the initial bifidobacterial counts of human volunteers or animals (Morishita et al., 1993; Van Loo et al., 1999). In spite of the diverse DP, EUC XOS, CC XOS and commercial XOS originated similar profiles of enhancement of bifidobacterial replication in the fermentations by the ileal inoculum (Fig. 4), and EUC XOS was able to maintain the bifidobacterial detection level further up to 72 h denoting some temporal coincidence with the respective consumption profile (Fig. 1). The maintenance of *Bifidobacterium* PCR titres at considerable detection levels up to 72 h in the case of EUC XOS (ileal digesta) and commercial XOS (colonic digesta) suggests that this bacterial group was able to attain a considerable representation throughout the fermentations, which is a desirable characteristic regarding XOS prebiotic potential.

The replication of *Bacteroides/Prevotella* occurred typically in a second stage of fermentation. In the fermentations by the ileal inoculum, PCR titres were inexpressive at 0 h but the values increased markedly from 11.5 to 30 h in all cases. The values were maintained or increased up to 72 h and were higher for XOS produced by autohydrolysis than for commercial XOS. With the caecal inoculum, *Bacteroides/Prevotella* PCR titres increased progressively up to 30 h in the fermentations of EUC XOS. Comparatively, this increase occurred only up to 11.5 h on commercial XOS.

CC XOS reached the highest BactrF1 + BactrF2 PCR titre at 30 h, whereas BSG XOS maximum was detected at 72 h. In the fermentations of commercial XOS and EUC XOS by the colonic inoculum the detection level of *Bacteroides/Prevotella* increased steadily up to 30 h and maintained the value up to 72 h. CC XOS produced a similar detection pattern except that the maximum PCR titre was only attained in the samples from 72 h. BSG XOS seemed unable to enhance replication of *Bacteroides/Prevotella* in the fermentations by colonic microorganisms. In order to compose some hypothesis concerning XOS capability to selectively stimulate the replication of beneficial bacteria it would be of utmost importance the establishment of a clear correlation between PCR up-shifts observed for *Bifidobacterium* and *Lactobacillus/Pediococcus* populations and XOS consumption profiles. Except in the fermentation of CC XOS by the colonic digesta, this correlation was not possible to set since the replication of these bacterial groups was mainly enhanced during the first 11.5 h. In fact, it was easier to identify a temporal coincidence between the highest XOS consumption rates and increases in PCR titres of *Bacteroides/Prevotella* populations than of bifidobacteria or lactobacilli, even for the ileal inoculum. *Bacteroides* strains are described as able to utilise commercial XOS (Crittenden et al., 2002). Consequently, the suggestion that medium- and long-chain XOS are not selective for bifidobacterial and/or lactobacilli growth would be consensual with previous observations for short-chain XOS. Considering that *Bacteroides/Prevotella* replication occurs mainly in a second stage of fermentation, it is not possible from this study to evaluate whether growth can be a direct result of XOS hydrolysis and uptake or of cross-feeding of intermediate substrates resulting from OS breakdown (Belenguer et al., 2006) or even from intermediate fermentation of lactate, as discussed above. More complete studies involving carbon flow analysis in controlled co-cultures would be necessary to enlighten these issues.

4. Conclusions

From this study, it can be concluded that XOS produced by autohydrolysis with a DP range up to 25 can be fermented *in vitro* by the ileal, caecal and colonic microbiota of a Duroc × Landrace piglet. The longer chain length of the XOS mixtures produced by autohydrolysis reduced notably the rate of XOS consumption in the fermentations by the microbiota from the piglet's ileum. EUC XOS, CC XOS and commercial XOS supported an enhancement of bifidobacteria and lactobacilli replication in a first stage of fermentation, up to 11.5 h. Apparently this stimulation was not selective, because *Bacteroides/Prevotella* replication increased in a second stage of fermentation, coincident in time with the highest consumption rates of some XOS mixtures tested. Mostly due to the slow fermentability by the ileal inocula, EUC XOS, CC XOS and BSG XOS can be regarded as promising functional candidates suitable to act as distally fermentable substrates. Amongst the mixtures produced by autohydrolysis, EUC XOS and CC XOS appeared to be more promising than BSG XOS in stimulating bifidobacteria and lactobacilli replication *in vitro*.

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