Population Dynamics of Some Relevant Intestinal Microbial Groups in Human Fecal Batch Cultures with Added Fermentable Xylooligosaccharides Obtained from Rice Husks

Lorena Valdés,[a] Patricia Gullón,[b] Nuria Salazar,[a,d] David Rios-Covián,[a] Maria Jesús González-Muñoz,[c] Juan Carlos Parajó,[c] Patricia Ruas-Madiedo,[a] Miguel Gueimonde,[a] and Clara G. de los Reyes-Gavilán [a,]*

Xylooligosaccharides (XOS) obtained by autohydrolysis of rice husks were demonstrated in a previous study to act as fermentable substrates by the intestinal microbiota in human fecal slurry cultures, leading to the generation of acetic and lactic acids and supporting the growth of bifidobacteria (Gullón *et al.* 2011). The purpose of the present study was to provide new insights into other possible targets of XOS action by determining (in the same fecal cultures) the levels of some relevant intestinal microbial groups and the profile of *Bifidobacterium* species by quantitative and qualitative polymerase chain reaction (PCR), respectively. XOS-containing concentrates promoted the growth of *Lactobacillus-Weissella*, *Bacteroides-Prevotella,* and *Clostridium* cluster XIVa groups, as well as *Faecalibacterium prausnitzii* species. Preliminary results point to possible variation in the profile of some bifidobacteria species in fecal cultures caused by XOS that should be further investigated. These results support XOS as potential prebiotics for the design of functional food products.

Keywords: Rice husks; Xylooligosaccharides; Fecal batch cultures; Bifidobacterium species; Lactobacillus; Bacteroides; Blautia coccoides-Eubacterium rectale; Faecalibacterium prausnitzii

Contact information: a: Department of Microbiology and Biochemistry of Dairy Products, Instituto de Productos Lácteos de Asturias, Consejo Superior de Investigaciones Científicas (IPLA-CSIC), Villaviciosa 33300, Asturias, Spain; b: CBQF/ Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal; c: Department of Chemical Engineering, Campus of Ourense, 32004, University of Vigo, Ourense, Spain; d: Metabolism and Nutrition Research Group, Louvain Drug Research Institute, Université Catholique de Louvain, Avenue E. Mounier 73, PO Box B1.73.11, 1200 Brussels, Belgium;
**Corresponding author: greyes_gavilan@ipla.csic.es*

INTRODUCTION

The human colonic microbiota play an important role in the maintenance of host health. Alterations in its composition and metabolic activity have been related to susceptibility to disease (Gerritsen *et al.* 2011). Prebiotics are defined as substrates that may improve host's health by selectively stimulating the growth and/or metabolic activity of one or a limited number of beneficial bacteria in the colon (Roberfroid *et al*. 2010). In order to be considered as prebiotics, these compounds must neither be hydrolyzed nor absorbed in the upper part of the intestinal tract (Gibson and Roberfroid 1995). Xylooligo-saccharides (XOS) can be considered NDOs, since they can pass undegraded

through the upper digestive tract and can arrive intact to the colon where they are fermented (Gullón *et al.* 2011; Mäkeläinen *et al.* 2010; Ohbuchi *et al.* 2009; Santos *et al.* 2006). XOS can be obtained from selected lignocellulosic materials containing substituted xylan, which are subjected to aqueous hydrolysis to solubilize the hemicelluloses and break down the polymeric xylan backbone. Further processing is necessary in order to use these oligosaccharides in food applications (Gullón *et al.* 2009).

Prebiotics have traditionally been related to a capacity to selectively stimulate the growth of bifidobacteria and lactobacilli populations in the intestinal microbiota. Recent evidence suggests that the prebiotic action may also involve intestinal microbial groups other than bifidobacteria and lactobacilli, the physiological characteristics of which are currently being investigated (Roberfroid *et al.* 2010).

In a previous study, using *in vitro* human fecal batch cultures, it was demonstrated that XOS generated by autohydrolysis of rice husks were fermented by the human colonic microbiota, producing a rapid decrease of pH, supporting the growth of bifidobacteria, and leading to the fast generation of acetic and lactic acids as the main products of fermentation (Gullón *et al.* 2011). The purpose of the present study is to gain insight into the impact of XOS on human intestinal microbiota by determining variations on other relevant members of such microbiota recognized as beneficial for human health: *Bacteroides-Prevotella*, saccharolytic microorganisms actively involved in degradation of complex carbohydrates, *Clostridium* clusters XIVa and IV, the main butyrate producers, and lactobacilli, some of whose strains have been traditionally considered as probiotics. Modifications in the composition of *Bifidobacterium* species which are normal inhabitants of the human gut have also been investigated. Results obtained with XOS were compared with those obtained with inulin, a recognized prebiotic, and with glucose, a sugar widely fermented by microorganisms but not considered as prebiotic, through the same *in vitro* approaches used previously (Gullón *et al*. 2011).

EXPERIMENTAL

Fermentation in Fecal Cultures with Added XOS Obtained from Rice Husks

XOS preparation

XOS were obtained from rice husks treated with hot, compressed water under specific conditions to cause the partial breakdown of xylan into soluble products (mainly XOS). Next, the reaction media were refined by membrane processing and ion exchange resins, with an intermediate endoxylanase treatment (XOS 2; DP 2-4) or without enzymatic treatment (XOS 1; degree of polymerization (DP) > 5-6) to obtain the purified XOS concentrates.

Fecal batch cultures

Non-pH controlled human fecal batch cultures with added XOS obtained from rice husks as the sole external carbon source were performed in anaerobic conditions at 37 ºC as specified in Gullon *et al.* (2011). Three fermentation trials with human fecal inocula (final concentration 1% w/v) corresponding to three healthy, adult donors (two women and one man, aged 25 to 40 years old, called individual 1, 2, and 3) were carried

out in independent duplicate experiments using XOS concentrates XOS 1 and XOS 2, inulin, and glucose (as positive selective and non-selective controls, respectively) as substrates (final concentration 1% w/v). Fecal batch cultures without an added external carbon source were used as negative controls.

Quantification of Intestinal Microbial Groups in Batch Cultures by qPCR

DNA extraction

One mL of fecal culture was employed for DNA extraction using the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) as indicated by Gullón *et al.* (2011).

qPCR reactions

Quantification by polymerase chain reaction (qPCR) of the levels of different bacterial populations was performed by duplicate in DNA extracted from fecal cultures with a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems). The primers and conditions indicated in Table 1 were used for *Bacteroides-Prevotella*, *Blautia coccoides-Eubacterium rectale* (*Clostridium* cluster XIVa), *Lactobacillus-Weissella* group, and *Faecalibacterium prausnitzii*. Standard curves were made with pure cultures of the appropriate strains. *Bacteroides thetaiotaomicron* DSMZ2079, *Blautia coccoides* DSMZ935, and *Lactobacillus gasseri* IPLAIF7/5 were grown in GAM medium (Nissui Pharmaceutical Co, Tokio, Japan) and *F. prausnitzii* DSMZ 17677 in RCM formula (Oxoid Ltd., Basingstoke Hampshire, UK) without agar, under anaerobic conditions at 37 ºC.

Determination of *Bifidobacterium* Species in Batch Cultures by PCR

In order to know the profile of *Bifidobacterium* species and whether XOS could promote variations in such profiles, the occurrence of different species from this genus was determined by qualitative PCR (UnoCycler, VWR International, Radnor, Pennsylvania, USA) using the primers and conditions indicated in Table 1.

RESULTS AND DISCUSSION

Changes in Intestinal Microbial Population Levels in Fecal Cultures

qPCR was used to evaluate the effect of XOS 1 and XOS 2 concentrates in fecal cultures compared with glucose and inulin. The response of intestinal microbial populations varied depending on the carbon source and donor, as was previously stated, regarding the bifidogenic effect and the production of short chain fatty acids (SCFA) (Gullón *et al.* 2011). Notable variations have been found in our previous work (Gullón *et al*. 2011) in SCFA concentrations among fecal cultures of the three individuals. Thus, fecal cultures of individual 1 who showed the lowest concentrations of SCFA displayed the highest levels of lactic acid and the fastest consumption of XOS whereas in contrast fecal cultures from individuals 2 and 3 presented higher levels of total SCFA and lower concentrations of lactic acid and slower consumption of XOS than individual 1. In the present work, the general behavioral pattern of intestinal populations in fecal cultures of

individual 1 also differed from that of individuals 2 and 3 (Fig. 1) and therefore, the results from fecal cultures of each donor were considered separately.

Table 1. Primers and Annealing Temperatures Used in this Study for Quantification by qPCR of Intestinal Microbial Groups and for Detection by Qualitative PCR of *Bifidobacterium* Species

Target	Primer sequence (5'-3')	Temp. (ºC)	PCR product length (bp)	Reference
Quantitative PCR:				
Lactobacillus-Weisella	F-AGCAGTAGGGAATCTTCCA R-CATGGAGTTCCACTGTCCTC	60	331	Arboleya *et al.* (2012)
Bacteroides-Prevotella	F-GAGAGGAAGGTCCCCCAC R-CGCKACTTGGCTGGTTCAG	60	108	Arboleya *et al.* (2012)
Bl. coccoides-E. rectale	F-CGGTACCTGACTAAGAAGC R-AGTTTYATTCTTGCGAACG	55	429	Arboleya *et al.* (2012)
F. prausnitzii	F-TAACACAATAAGTWATCCACCTGG R-ACCTTCCTCCGTTTTGTCAAC	60	248	Ramirez-Farias *et al.* (2009)
Qualitative PCR:				
B. longum group	F-TTCCAGTTGATCGCATGGTCTTCT R-GGCTACCCGTCGAAGCCACG	65	110	Gueimonde *et al.* (2007)
B. pseudocatenolatum group	F-GCCGGATGCTCCGACTCCT R-ACCCGAAGGCTTGCTCCCGAT	64	300	Gueimonde *et al.* (2007)
B. breve	F-AATGCCGGATGCTCCATCACAC R-GCCTTGCTCCCTAACAAAAGAGG	66	285	Gueimonde *et al.* (2007)
B. adolescentis group	F-CTCCAGTTGGATGCATGTC R-CGAAGGCTTGCTCCCAGT	61	279	Matsuki *et al.* (2004)
B. bifidum	F-TGACCGACCTGCCCCATGCT R-CCCATCCCACGCCGATAGAAT	61	110	Gueimonde *et al.* (2007)
B. dentium	F-ATCCCGGGGGTTCGCCT R-GAAGGGCTTGCTCCCGA	55	387	Matsuki *et al.* (2004)
B. angulatum	F-CAGTCCATCGCATGGTGGT R-GAAGGCTTGCTCCCCAAC	59	275	Matsuki *et al.* (2004)

Lactobacillus-Weissella group

All substrates assayed resulted in a noticeable increase (at least 1 log unit) of *Lactobacillus-Weissella* levels in fecal cultures of individual 1, compared with the negative control culture. This increase was more pronounced at 6 h of incubation with XOS concentrates than with glucose or inulin. In contrast, in the fecal cultures of the

other two individuals (2 and 3), who presented initial levels of lactobacilli 2 to 3 log units lower than individual 1, counts of this microbial group suffered only minor variations during incubation with all substrates. Gullón *et al.* (2011) reported higher initial levels of bifidobacteria and a lower bifidogenic effect of XOS substrates in cultures from donor 1 than in cultures from the other two individuals. The opposite seems to occur in the present work with lactobacilli, since fecal cultures from the individual having the highest initial levels also displayed the most pronounced effect of XOS on this microbial group. In this regard, it has been considered that, when referring to bifidobacteria, the major determinant that quantitatively controls the prebiotic effect is the initial number that the volunteers have in feces before supplementation with the compound to be tested (Kolida *et al.* 2007; Roberfroid *et al.* 2010). Bifidobacteria and lactobacilli are lactic acid producers; the higher levels of this acid previously reported by us in fecal cultures of individual 1 with respect to the other two donors are consistent with the higher initial levels of bifidobacteria and lactobacilli (Gullón *et al.* 2011). The increase of lactobacilli and bifidobacteria has been recently reported by Muralikrishna *et al*. (2011) in fecal cultures added feruloyl-XOS obtained from wheat bran.

Bacteroides-Prevotella group

Counts of *Bacteroides-Prevotella* showed moderate increases (up to 0.5 log units in most cases) over the negative control during fermentation with glucose, inulin, or XOS in fecal cultures from the three donors; no noticeable differences in the initial levels of this microbial group were found among individuals (Fig. 1). Growth of *Bacteroides-Prevotella* has previously been reported in *in vitro* fermentation of selected XOS by piglet intestinal microbiota (Moura *et al.* 2008) and in feces of mice that were fed XOS (Petersen *et al.* 2010). However, a decrease of this microbial group was found in human fecal cultures added feruloyl-XOS from wheat bran and XOS from birch wood (Muralikrishna *et al*. 2011). Differences in composition between XOS and feruloyl-XOS as well as on intestinal microbiota of humans and animals could be on the basis of the different response against these substrates of *Bacteroides-Prevotella* group reported by different authors. Members of the *Bacteroides* group are saccharolytic and are among the predominant genera in the gut of mammals (Faust *et al.* 2012). The increase of *Bacteroides-Prevotella* in fecal cultures of the three individuals as evidenced in the present study suggests that XOS are fermented by these microorganisms. The slight production of propionic acid in the current fecal cultures (Gullón *et al.* 2011) is in agreement with the capability of *Bacteroides* to produce variable amounts of propionic acid, depending on the culture conditions (Macfarlane and Gibson 1997).

Clostridium cluster XIVa and Faecalibacterium prausnitzii

Most butyrate-producing intestinal bacteria are included in *Clostridium* clusters XIVa and IV (Barcenilla *et al.* 2000), which are among the predominant microorganisms present in the intestinal microbiota of healthy individuals. Indeed, high levels of *F. prausnitzii*, belonging to the *Clostridium* cluster IV, have been related to colonic fiber fermentation and production of butyrate in healthy individuals (Benus *et al.* 2010). Conversely, several diseases have been found to decrease the population counts of *F. prausnitzii* (De Palma *et al.* 2010; Candela *et al.* 2012; Li *et al.* 2012). Butyrate produced

by the activity of intestinal bacteria is metabolized quickly by the gut epithelium and/or the liver, promoting beneficial effects in host health (Guilloteau *et al.* 2010; Canani *et al*. 2011).

In our previous work there was evidence of a moderate increase of butyrate in human fecal cultures added with XOS (Gullón *et al*. 2011). This prompted us to study the dynamics of the butyrate-producers *Clostridium* cluster XIVa and the species *F. prausnitzii* in our experimental conditions. XOS, as well as glucose and inulin, supported growth (less than 1 log unit) of *F. prausnitzii* in fecal cultures of the three donors compared to the negative control cultures (Fig. 1). These results were similar to those found for this species by Muralikrishna *et al*. (2011) when using feruloyl-XOS as carbon sources in human fecal cultures.

Inulin promoted growth of *Bl. coccoides-E. rectale* in fecal cultures of individuals 1 and 3 with respect to the negative controls, whereas glucose had that effect on the cultures of donor 2. XOS 2, but not XOS 1, slightly stimulated the growth of *Clostridium* cluster XIVa in fecal cultures of individuals 2 and 3. The enhancement of *Bl. coccoides-E. rectale* growth by XOS and FOS was previously reported by Petersen *et al.* (2010). Remarkably, in the fecal cultures in this work, the slight increase of *Clostridium* cluster XIVa population levels was coincident with a slightly higher production of butyrate when XOS 2 was added. The fermentation of XOS 2 by *Bl. coccoides-E. rectale* points to a preferential stimulation of this group in fecal cultures of individuals 2 and 3 by this substrate. This is probably related to the lower DP and easier use as a fermentable substrate of XOS 2 over XOS 1.

In summary, the above results indicate that XOS used as fermentable substrates are able to stimulate microbial populations other than bifidobacteria in fecal cultures. Levels of *Bacteroides-Prevotella* and *F. prausnitzii* increased in fecal cultures of the three individuals tested. However, the behavior of the other analyzed microbial groups fit into two different patterns.

In the case of one individual, the growth of *Lactobacillus-Weissella* but not *Clostridium* cluster XIVa was stimulated when using XOS as fermentable substrates. In contrast, in the fecal cultures of the other two individuals, the presence of XOS caused a slight increase of *Clostridium* cluster XIVa without increases of the *Lactobacillus* group. A different response to fermentable carbohydrates depending on the individual was reported in human intervention studies (Davis *et al.* 2010), as well as in fecal cultures using different carbohydrate substrates (Salazar *et al.* 2008, 2009). In this regard, when referring to bifidobacteria, the initial number of targeted bacteria per gram of feces has been considered to be the major determinant that quantitatively controls the prebiotic effect (Kolida *et al.* 2007; Roberfroid *et al.* 2010).

In the present study the initial counts of the *Lactobacillus-Weissella* population in each donor could have been a key factor in the different responses to the carbohydrates tested; in fact, an antagonistic behavior seems to occur between lactobacilli and *Clostridium* cluster XIVa in our experimental conditions. A key aspect considered on prebiotics is selectivity towards lactobacilli and bifidobacteria. However, given the complexity of interactions occurring among microbial intestinal groups and fermentable carbohydrates in the human gut, it has been suggested broadening the prebiotic concept to some other microbial groups (FAO 2007; Roberfroid *et al*. 2010).

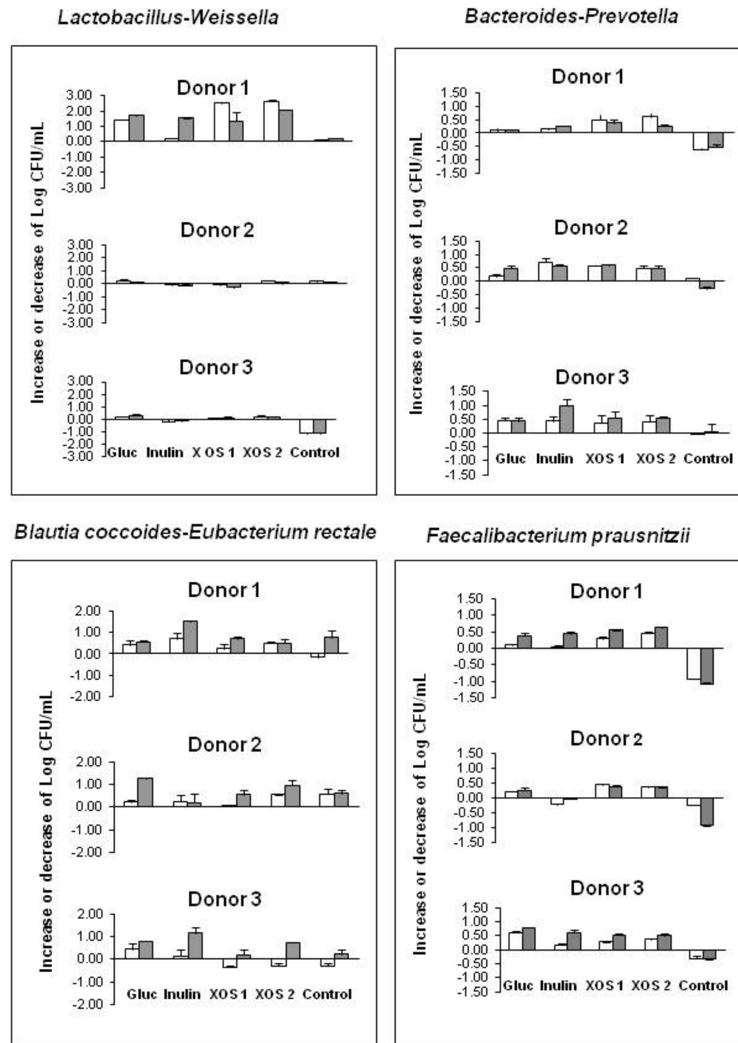

Fig. 1. Variations (with respect to time 0 h) of *Lactobacillus-Weissella*, *Bacteroides-Prevotella*, *Blautia coccoides-Eubacterium rectale* (*Clostridium* cluster XIVa) groups, and *Faecalibacterium prausnitzii* species counts determined by qPCR in fecal slurry cultures from three donors, using XOS 1, XOS 2, glucose, or inulin as carbon sources. Data was obtained after 6 h (white bars) and 24 h (grey bars) of incubation. The control did not contain any added carbohydrates. Trial for each individual reflects the result of independent duplicate experiment, and each PCR run was carried out twice. Error bars indicate standard deviation for these four values. Initial (time 0) counts of microbial groups in fecal cultures as depending on donors were as follows: *Lactobacillus-Weissella*: 7.13 ± 0.01 log CFU/g for donor 1, 4.83 ± 0.03 for donor 2, and 5.10 ± 0.07 for donor 3, *Bacteroides-Prevotella*: 8.31 ± 0.02 CFU/g for donor 1, 8.36 ± 0.06 for donor 2, and 8.40 ± 0.12 for donor 3, *Bl. coccoides-E. rectale*: 7.36 ± 0.16 CFU/g for donor 1, 7.34 ± 0.00 for donor 2, and 8.04 ± 0.06 for donor 3, *F. prausnitzii*: 6.60 ± 0.05 CFU/g for donor 1, 6.70 ± 0.06 for donor 2, and 6.75 ± 0.09 for donor 3.

Profile of *Bifidobacterium* Species in Fecal Cultures

Qualitative PCR evidenced the presence of the species *B. adolescentis* in all fecal cultures from the three individuals, whereas *B. angulatum* was absent. Table 2 shows variations in the presence of the other *Bifidobacterium* species. *B. longum* was detected in most fecal cultures. Detection of *B. pseudocatenulatum* and *B. bifidum* showed variation among donors. No remarkable changes in the presence of these microorganisms were found during the incubation of fecal cultures with different carbon sources. The species *B. breve* was not initially detected in any of the samples; interestingly, after 24 h of incubation, fecal cultures from individual 1 became positive for *B. breve* in glucose, XOS 1, and XOS 2. *B. dentium* appeared in control cultures of donor 3 at 0 and 24 h of incubation, whereas the donor became detectable for this species only in the presence of inulin when feces were incubated with the different carbon sources (Table 2).

Table 2. Presence (+) or Absence (-) Determined by Qualitative PCR at 0 and 24 h of Incubation of some *Bifidobacterium* Species in Human Fecal Cultures with Added Glucose, Inulin, XOS 1, or XOS 2 and in Fecal Cultures Without External Carbon Sources Added (Control)

		Donor 1		Donor 2		Donor 3	
		0 h	24 h	0 h	24 h	0 h	24 h
B. longum							
	Control	+	+	+	+	_	+
	Glucose	+	+	+	+	+	+
	Inulin	+	+	+	+	+	+
	XOS 1	+	+	+	+	+	+
	XOS 2	+	+	+	+	+	-
B. breve							
	Control	-	-	-	-	-	-
	Glucose	-	+	-	-	-	-
	Inulin	-	-	-	-	-	-
	XOS 1	-	+	-	-	-	-
	XOS 2	-	+	-	-	-	-
B. bifidum							
	Control	-	-	-	-	-	-

	Glucose	+	+	-	-	-	-
	Inulin	-	-	-	-	+	+
	XOS 1	-	-	-	-	-	-
	XOS 2	-	-	-	-	-	-
B. dentium							
	Control	-	-	-	-	+	+
	Glucose	-	-	-	-	-	-
	Inulin	-	-	-	-	-	+
	XOS 1	-	-	-	-	-	-
	XOS 2	-	-	-	-	-	-
B. pseudocatenulatum							
	Control	+	+	+	+	-	-
	Glucose	+	+	+	+	-	-
	Inulin	+	+	+	+	-	-
	XOS 1	+	+	+	+	-	-
	XOS 2	+	+	+	+	-	-

Population levels close to the detection limit could account for the inconsistency found in the results at time 0 with fecal cultures from donors 1 and 3 with the different carbon sources for the species *B. longum*, *B. bifidum,* and *B. dentium*. Although the low number of individuals and samples analyzed does not allow any conclusions to be reached, the results should spark interest in further investigation into the possible influence of XOS in the profile of bifidobacteria species in fecal cultures. Related to this, there has recently been a report in human intervention studies on the enrichment of particular bifidobacteria species in human fecal samples after the intake of inulin-type fructans and green tea (Joossens *et al.* 2011; Jin *et al.* 2012). The different abilities of species to directly ferment prebiotic substrates or to interact through cross-feeding mechanisms with other members of the intestinal microbiota could explain the different behavior of the species of *Bifidobacterium* against these compounds (Pastell *et al.* 2009; Saarinen *et al.* 2012).

Some current studies deal with the prebiotic effect of XOS from different origins and DP (Broekaert *et al.* 2011), but only a few of them have analyzed the effect of these

NDOs on intestinal microbial communities (other than bifidobacteria) either *in vitro* or *in vivo* (Moura *et al.* 2008; Petersen *et al.* 2010). The present study provides new insight on *in vitro* intestinal microbial population dynamics as affected by XOS as fermentable substrates in fecal cultures.

CONCLUSIONS

1. When used as fermentable carbohydrates in human fecal batch cultures, XOS concentrates promoted growth of *Lactobacillus-Weissella*, *Bacteroides-Prevotella*, and *Clostridium* cluster XIVa groups, as well as *F. prausnitzii* species.
2. Preliminary results suggest possible variations on the profile of certain bifidobacteria species after fecal cultures were incubated with XOS.
3. Knowledge of the effect that specific NDOs exert on certain intestinal microbial groups in different individuals could allow the design of specific functional food products containing XOS.

ACKNOWLEDGMENTS

The authors acknowledge financial support from Plan Nacional de I+D through the Project AGL2010-16525, which partially received funds from FEDER Program of the European Union. Lorena Valdés was the recipient of a predoctoral fellowship JAE-pre from CSIC. Nuria Salazar acknowledges her past FPI fellowship from the Spanish Ministry of Science and Innovation and her current postdoctoral fellowship from the Spanish Ministry of Education. Patricia Gullón wishes to thank her financial support provided by postdoctoral fellowships (SFRH/BPD/79942/2011) issued by FCT (Fundação para a Ciência e Tecnologia).

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Article submitted: January 16, 2013; Peer review completed: March 19, 2013; Revised version received and accepted: March 26, 2013; Published: March 28, 2013.