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Prebiotic potential of novel carbohydrates in an in vitro co-inoculation fermentation model of the bacteria isolated from pig intestine and *Salmonella*

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ABSTRACT: Innovative plant biomass fractionation methods produce new feed additives that could modulate pig intestinal microbial communities. Such novel indigestible carbohydrates (CHO) were investigated for their prebiotic potential and their influence on *Salmonella* Typhimurium in a co-inoculation in vitro fermentation model of pig intestines. Inulin, cellobiose, pectic oligosaccharides (POS), isomalto-oligosaccharides (IMO), xylooligosaccharides (XOS), and gluconic acid (GLU) were fermented for 72 h by a bacterial fecal inoculum from pigs in an in vitro model in the presence of *Salmonella*. Gluconic acid was the fastest fermenting CHO followed by inulin and IMO ($P = 0.01$). After 6 h, cellobiose yielded the highest lactate molar ratio (0.484). Pectic oligosaccharides fermented more slowly. Xylooligosaccharides and GLU were little fermented (150 and 175 mg short-chain fatty acids/g after 24 h). Nonetheless, GLU yielded the highest butyrate molar ratio of all CHO (0.290 at 12 h; $P < 0.01$). Although *Salmonella* counts did not differ, inulin and IMO displayed prebiotical properties, because they supported the highest *Lactobacillus* and *Bifidobacterium* populations after 12 and 24 h of fermentation (7.38 to 8.86 log cfu/mL; $P < 0.01$). Cellobiose and GLU scored well for *Lactobacillus* too but poorly supported *Bifidobacterium* (6.41 to 6.92 log cfu/mL; $P < 0.01$). It is concluded that IMO seems the most promising prebiotic, but owing to their specific fermentation patterns, cellobiose and GLU deserve further consideration.

Key words: indigestible carbohydrates, in vitro fermentation, *Salmonella*

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INTRODUCTION

Salmonella enterica subsp. *enterica*, particularly *Salmonella* Typhimurium, is an important foodborne pathogen transmitted by pigs (EFSA, 2013), and nutritional factors can contribute to reduce *Salmonella* prevalence in pigsties (O'Connor et al., 2008). Prebiotics in the form of nondigestible carbohydrates (**CHO**) have been reported to restore or improve the resistance to colonization, and the intestinal barrier function against invading pathogens is thereby reinforced (Bindels et al., 2015). Innovative biomass fractionation methods (Combo et al., 2013) were applied on sugar beet pulp and wheat bran yield novel oligosaccharides such as isomalto-oligosaccharides (**IMO**), pectic oligosaccharides (**POS**), and xylooligosaccharides (**XOS**) that were compared with gluconic acid (**GLU**), inulin, and cellobiose for their potential to modulate the intestinal ecophysiology using an in vitro model of the pig gastrointestinal tract. The model was run under a *Salmonella* Typhimurium challenge (Pieper et al., 2009).

MATERIALS AND METHODS

Ingredients

Six ingredients were used: cellobiose and GLU (Sigma-Aldrich, Belgium); POS, IMO, and XOS (Table 1), provided by the Department of Industrial Biological Chemistry (Gembloux Agro-Bio Tech, Gembloux, Belgium); and inulin (Fibruline Instant; Cosucra, Warcoing, Belgium) as a reference prebiotic. Total CHO and free sugar contents were 0.790 and <20 g/kg for IMO, 0.327 and 36 g/kg for POS, and 0.532 and 131 g/kg for XOS, respectively.

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Table 1. Gas production kinetics parameters of the fermentation by porcine feces of carbohydrates in the presence of *Salmonella* Typhimurium (n = 3)

Ingredient[1]	A,[2] mL/g	B,[3] h	R_{max},[4] mL·h^{-1}·g^{-1}	T_{max},[5] h
Inulin	278[b]	6.51[b]	35.1[a]	5.1[b]
Cellobiose	289[b]	7.12[b]	33.2[a]	5.6[b]
IMO	262[b]	7.08[b]	28.0[a]	5.2[b]
POS	172[c]	7.19[b]	16.2[b]	4.7[b]
XOS	59[d]	5.33[c]	13.5[b]	4.4[b]
GLU	329[a]	8.52[a]	37.0[a]	7.2[a]
SEM	95	1.00	9.81	1.00
P-values	<0.001	<0.001	<0.001	0.004

[a–c]Means followed by different letters within a column differ significantly ($P < 0.05$).

[1]IMO = isomalto-oligosaccharides; POS = pectic oligosaccharides; XOS = xylooligosaccharides; GLU = gluconic acid.

[2]A = asymptotic gas production.

[3]B = mid-fermentation time.

[4]R_{max} = maximum rate of gas production.

[5]T_{max} = time at which the R_{max} is reached.

In Vitro Batch Co-Inoculation and Analyses

Carbohydrates (200 mg) were fermented in triplicates with 3 incubation times (12, 24, and 72 h) by a fecal inoculum (30 mL) prepared with feces mixed from 3 *Salmonella*-free sows (Belgian Landrace × Pietrain) of the herd of the Walloon Agricultural Research Center (Gembloux, Belgium) diluted 20 times on a weight basis in a carbonate buffer providing macro- and microminerals (Menke and Steingass, 1988), with the addition of 6 mucin-covered microcosms per fermentation flask (approximately 1 g of mucus agar; Van den Abbeele et al., 2012). After a 6-h period of adaptation to the ingredients to mimic pathogen contamination in an established microbial community in the intestines as described in Pieper et al. (2009), each bottle was infected with a suspension of *Salmonella* Typhimurium (CWBI-B1501; Gembloux, Belgium) to obtain a *Salmonella* concentration of 2×10^7 cfu/mL (log 7.3) in the bottles. Blank bottles, devoid of CHO, were also included as control. When stopped after 12 and 24 h, fermentation broth was centrifuged (12,000 × *g* for 5 min) and the supernatant used for short-chain fatty acids (**SCFA**) analysis using a Waters 2690 HPLC system (Waters Corp., Milford, MA). Branched-chain fatty acids (**BCFA**) were calculated as the sum of *i*-butyrate, *n*-valerate, and *i*-valerate, and total SCFA were calculated as the sum of acetate, propionate, *n*-butyrate, and BCFA. The centrifuged pellet was used for extraction of microbial genomic DNA using a QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) preceded by a bead beating step (FastPrep-24; MP Biomedicals, Illkirsh, France). Quantitative real-time PCR was performed as described in Boudry et al. (2012) using a StepOnePlus sequence detection system (Applied Biosystems, Halle, Belgium).

Calculations and Statistical Analyses

Cumulative gas pressure measurements for 72 h converted in volume were modeled according to (Groot et al., 1996):

$$G_i = (A \times t_i^C)/(t_i^C + B^C),$$

in which G_i (mL/g) represents the total cumulative gas, t_i (h) represents the incubation time, A (mL/g) represents the asymptotic gas production, **B** (h) represents the mid-fermentation time, and C (–) represents the switching sigmoidal characteristics of the curve. The maximum rate of gas production ($\mathbf{R_{max}}$; mL·h^{-1}·g^{-1}) and the time at which the R_{max} is reached ($\mathbf{T_{max}}$; h) were calculated from model's equation.

Data were analyzed for homogeneity of variances with a Bartlett test and log transformed where required. The influence of the ingredient and/or time of incubation and their interactions on the observations were assessed using a 1- or 2-way ANOVA model in R 3.1.0 software (The R Foundation, Vienna, Austria) and means were compared using a Student–Newman–Keuls post hoc test.

RESULTS AND DISCUSSION

Xylooligosaccharides were fermented very little (A = 59 mL/g and 150 mg SCFA/g after 24 h), although, owing to its high free sugars content, it was the fastest fermenting CHO with earlier B (5.33 h) followed by inulin, IMO, cellobiose, and POS ($P < 0.001$; Tables 1 and 2). Gluconic acid was the latest fermenting CHO (B = 8.52 h and T_{max} = 7.2 h; $P < 0.001$) but generated higher final gas production (A = 329 mL/g) and R_{max} (37.0 mL·h^{-1}·g^{-1}). Surprisingly, the low SCFA production of GLU (175 mg SCFA/g after 24 h) did not match with its high gas production, possibly due to fermentation pathways that produce higher fermentation gases (CO_2 and CH_4) and to a direct release of CO_2 from the carbonate to buffer its acidity. Gluconic acid, however, yielded the highest butyrate molar ratio (0.290 at 12 h; $P < 0.01$). After 6 h (data not shown), cellobiose yielded the highest SCFA production and lactate molar ratio (0.484) followed by inulin (0.275) and IMO (0.172), making those CHO probably interesting for microbial community regulation in the ileum. High SCFA productions of cellobiose did not inhibit *Salmonella* growth (Table 3), probably because the carbonate buffer prevented proper acidification by the SCFA. However, in vivo, high butyrate production observed with cellobiose

Table 2. Short-chain fatty acid (SCFA) production and molar ratios according to the fermentation time by porcine feces of carbohydrates in the presence of *Salmonella* Typhimurium ($n = 3$)

Time, h	Ingredient[1]	SCFA, mg/g	Acetate, %	Propionate, %	Butyrate, %	BCFA,[2] %
12	Inulin	511.4^{a}	50.5^{de}	30.3^{ab}	9.3^{bc}	9.8^{b}
	Cellobiose	494.4^{a}	46.6^{e}	38.0^{a}	8.8^{bc}	6.5^{b}
	IMO	462.0^{a}	54.3^{cd}	25.6^{abc}	11.9^{bc}	7.7^{b}
	POS	441.8^{a}	67.4^{ab}	16.4^{bc}	6.9^{bc}	9.3^{b}
	XOS	103.0^{b}	65.6^{ab}	27.9^{abc}	1.2^{c}	4.7^{b}
	GLU	139.0^{b}	53.9^{d}	0.9^{d}	29.4^{a}	15.7^{a}
24	Inulin	514.1^{a}	54.0^{d}	31.8^{ab}	8.2^{bc}	6.0^{b}
	Cellobiose	545.3^{a}	54.6^{d}	25.3^{abc}	12.5^{bc}	7.5^{b}
	IMO	549.9^{a}	57.4^{c}	25.0^{abc}	10.4^{bc}	7.1^{b}
	POS	547.1^{a}	69.1^{a}	19.7^{bc}	6.0^{bc}	5.3^{b}
	XOS	149.4^{b}	63.8^{ab}	24.8^{abc}	5.6^{bc}	5.6^{b}
	GLU	174.5^{b}	62.0^{bc}	12.5^{c}	16.7^{b}	8.5^{b}
SEM		185	7.7	10.7	7.3	3.8
P-values						
Ingredient		<0.001	<0.001	<0.001	<0.001	0.013
Time		0.004	<0.001	0.970	0.314	0.035
Ingredient × time		0.637	0.05	0.037	0.015	0.139

$^{a–d}$Means followed by different letters within a column differ significantly ($P < 0.05$).

[1]IMO = isomalto-oligosaccharides; POS = pectic oligosaccharides; XOS = xylooligosaccharides; GLU = gluconic acid.

[2]BCFA = branched-chain fatty acids.

might decrease pathogenicity through downregulation of the expression of genes located on the *Salmonella* pathogenicity island 1 (Gantois et al., 2006).

Some CHO displayed obvious prebiotical properties, namely inulin and IMO, because they supported high *Lactobacillus* and *Bifidobacterium* populations after 12 h (8.18 to 8.56 log cfu/mL; $P < 0.01$). Cellobiose and GLU scored well for *Lactobacillus* too but poorly supported *Bifidobacterium* growth (6.89 to 6.92 log cfu/mL; $P < 0.01$), but all this did not impact *Salmonella* counts, similar to observations by Martín-Peláez et al. (2008) with other CHO sources.

Table 3. Bacteria counts in the fermentation broth measured by quantitative real-time PCR ($n = 3$)

Time, h	Ingredient[1]	*Salmonella*, log cfu/mL	*Lactobacillus*, log cfu/mL	*Bifidobacterium*, log cfu/mL	*Clostridium*, log cfu/mL	*Bacteroides*, log cfu/mL
12	Control	7.46^{ab}	7.96^{bc}	6.56^{d}	6.72	9.77^{fg}
	Inulin	7.56^{ab}	8.38^{a}	8.18^{ab}	6.76	10.58^{ab}
	Cellobiose	7.57^{ab}	8.31^{ab}	6.81^{d}	7.02	10.40^{c}
	IMO	7.62^{a}	8.46^{a}	7.86^{abc}	6.79	10.48^{bc}
	POS	7.59^{ab}	8.12^{abc}	7.11^{cd}	6.82	10.63^{a}
	XOS	7.59^{ab}	8.14^{abc}	7.40^{bcd}	6.78	10.28^{d}
	GLU	7.60^{ab}	8.19^{abc}	6.83^{d}	6.83	9.88^{ef}
24	Control	7.49^{ab}	7.96^{bc}	6.52^{d}	6.87	9.78^{fg}
	Inulin	7.57^{ab}	8.11^{abc}	8.17^{ab}	6.87	10.43^{c}
	Cellobiose	7.52^{ab}	7.85^{c}	6.60^{d}	6.79	10.28^{d}
	IMO	7.49^{ab}	7.85^{c}	7.86^{abc}	6.74	10.24^{d}
	POS	7.40^{ab}	7.78^{c}	7.18^{cd}	6.76	10.23^{d}
	XOS	7.40^{ab}	7.88^{c}	7.00^{cd}	6.75	9.93^{e}
	GLU	7.37^{b}	7.84^{c}	6.41^{d}	6.69	9.70^{g}
SEM		0.11	0.25	0.77	0.16	0.32
P-values						
Ingredient		0.321	0.015	<0.001	0.772	<0.001
Time		<0.001	<0.001	0.056	0.492	<0.001
Ingredient × time		0.050	0.045	0.615	0.437	<0.001

$^{a–g}$Means followed by different letters within a column differ significantly ($P < 0.05$).

[1]IMO = isomalto-oligosaccharides; POS = pectic oligosaccharides; XOS = xylooligosaccharides; GLU = gluconic acid.

This can be explained by the fact that, under in vitro conditions as stated before, the expression of antimicrobial activity of these genera was not robust enough to inhibit *Salmonella*. *Salmonella* is a saccharolytic Enterobacteriaceae, which can ferment CHO and was able to survive in the buffered system and occupy an ecological niche (Pieper et al., 2009). Another explanation could lie in the stimulation of some resident gut microbes in fermenting CHO and producing metabolites and microbial proteins that *Salmonella* could use for its own growth (Petersen et al., 2009).

It is concluded that none of the investigated CHO could establish a microbial population inhibiting *Salmonella* growth in the buffered medium. However, IMO seem promising for its positive influence on microbial communities, but owing to their specific fermentation patterns, cellobiose and GLU also deserve further investigation in in vivo models.

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