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Research Article

RESEARCH ARTICLE

Adding mucins to an *in vitro* batch fermentation model of the large intestine induces changes in microbial population isolated from porcine feces depending on the substrate

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One sentence summary: Mucus supports better some bacteria from the intestines in an *in vitro* model of the pig intestine no matter if cellulose or inulin is fermented.

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ABSTRACT

Adding mucus to *in vitro* fermentation models of the large intestine shows that some genera, namely lactobacilli, are dependent on host–microbiota interactions and that they rely on mucosal layers to increase their activity. This study investigated whether this dependence on mucus is substrate dependent and to what extent other genera are impacted by the presence of mucus. Inulin and cellulose were fermented *in vitro* by a fecal inoculum from pig in the presence or not of mucin beads in order to compare fermentation patterns and bacterial communities. Mucins increased final gas production with inulin and shifted short-chain fatty acid molar ratios ($P < 0.001$). Quantitative real-time PCR analyses revealed that *Lactobacillus* spp. and *Bifidobacterium* spp. decreased with mucins, but *Bacteroides* spp. increased when inulin was fermented. A more in-depth community analysis indicated that the mucins increased Proteobacteria (0.55 vs 0.25%, $P = 0.013$), Verrucomicrobia (5.25 vs 0.03%, $P = 0.032$), *Ruminococcaceae*, *Bacteroidaceae* and *Akkermansia* spp. Proteobacteria (5.67 vs 0.55%, $P < 0.001$) and *Lachnospiraceae* (33 vs 10.4%) were promoted in the mucus compared with the broth, while *Ruminococcaceae* decreased. The introduction of mucins affected many microbial genera and fermentation patterns, but from PCA results, the impact of mucus was independent of the fermentation substrate.

Keywords: mucins; *in vitro* model; intestinal fermentation; inulin; cellulose; microbiota

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INTRODUCTION

Dietary fiber (DF) is commonly defined as plant polysaccharides that are resistant to hydrolysis by digestive secretions. Under this definition fall a wide range of carbohydrates (CHOs) known as non-starch polysaccharides (NSPs), which include pectins, cellulose, hemicelluloses, β-glucans and fructans, but also resistant starch and oligosaccharides. In non-ruminant species, including humans and pigs, these CHOs resist digestion in the upper gastrointestinal tract (GIT) and reach the ileum and the colon where they undergo fermentation by resident microbes. Because of their differences in monomeric composition, links, chain length and side-chains, these different indigestible CHOs are fermented by different bacterial species and yield different kinds of microbial metabolites, and hence their direct and indirect impact on the microbial population, intestinal ecophysiology and health (Roberfroid *et al.* 2010).

Boudry *et al.* (2012) showed that some bacterial genera respond in a similar pattern when they ferment pure indigestible CHOs, namely inulin and cellulose, in *in vivo* and *in vitro* models while the growth of others, namely lactobacilli, is lower *in vitro* than *in vivo* when comparing microbial populations in different sections of the intestinal tract of pigs. This difference highlights the potential dependence on the mucosal layers in the establishment of some microbial communities in the intestines when fermenting different types of CHO. Hence, thanks to their experimental reductionism, *in vitro* models allow the investigation of host–microbiota interactions (Venema and van den Abbeele 2013), specifically the role of mucosal layers, in which specific microbial populations proliferate, such as members of the *Bacteroides fragilis* group, *Enterobacteriaceae*, *Clostridiaceae* (Macfarlane, Woodmansey and Macfarlane 2005; Wüst, Horn and Drake 2011), and lactobacilli (Van Tassell and Miller 2011). Indeed, mucins are heavily glycosylated proteins with high molecular mass, produced by the epithelial cells and secreted in the gut. They concentrate on the outer part of the intestinal wall where they give the mucus its slimy structure and act as an adhesion site for some bacterial species (Macfarlane, Woodmansey and Macfarlane 2005), particularly *Lactobacillus* sp. (Van Tassell and Miller 2011). To understand the dependence of some microbes on mucus, porous polysaccharide gel beads (Cinquin *et al.* 2004, 2006), mucin beads confined within a dialysis membrane (Probert and Gibson 2004), or mucin-coated plastic carriers have been added in fermentation models (Van den Abbeele *et al.* 2012). Interestingly, in the presence of these adhesion supports, the number of bacteria is higher, especially lactobacilli (Cinquin *et al.* 2004, 2006; Van den Abbeele *et al.* 2012). However, it is worth questioning whether the presence of mucus also plays a role in the survival of a wider number of bacterial genera and species than lactobacilli and whether this dependence on mucus is influenced by the fermented ingredient. For this reason, in this study, two contrasted indigestible CHOs, inulin and cellulose, were fermented by a fecal inoculum in an *in vitro* batch model of the pig's large intestine in the presence or not of mucin beads in order to compare fermentation patterns as well as the changes in bacterial populations during fermentation of both ingredients.

MATERIALS AND METHODS

Ingredients

Two ingredients were used: inulin (Fibruline Instant, Cosucra, Warcoing, Belgium) and cellulose (Alba-fibre cellulose, Mikro-Technik Gmbh & Co. KG, Burgstadt, Germany).

Preparation of mucin-covered microcosms

Mucin-covered microcosms (MCMs) were prepared as described by Van den Abbeele *et al.* (2012). Briefly, plastic carriers (length = 7 mm, diameter = 9 mm, specific surface area = 800 $m^2.m^{-3}$, AnoxKaldnes K1 carrier, AnoxKaldnes AB, Lund, Sweden) were coated with mucus by soaking them in a solution (pH 6.8) of 5% porcine mucus type II (Sigma, Bornem, Belgium) and 1% agar.

In vitro batch fermentation

In vitro fermentation was performed as described by Bindelle *et al.* (2011). Briefly, a fecal inoculum was prepared by diluting 20 times (w/v) fresh feces samples taken from three sows of the herd of the Walloon Agricultural Research Center (Gembloux, Belgium) in a buffer solution (Menke and Steingass 1988) kept at 39°C under continuous bubbling of CO_2 to ensure steady anaerobic conditions. Two hundred milligrams of one of the ingredients was placed into 140 ml glass bottles equipped with pressure sensors and connected to a computer (AnkomRF Gas Production System, Ankom Technology, NY, USA). Six MCMs making up approx. 1 g of mucus–agar were placed in half of the bottles. Fermentation started when 30 ml of a freshly prepared fecal inoculum was poured in each bottle and the bottle was closed with the sensor head after flushing with CO_2, and put in an shaking incubator (39°C, 50 r.p.m.) for 72 h. During incubation, the headspace pressure of each bottle was recorded every 5 min. Blank bottles, devoid of ingredient, with and without MCM, were also included as controls to correct measurements performed during fermentation. Inoculum samples were also taken for the same purpose. The experimental scheme was as follows: 3 ingredients (inulin, cellulose, control) × 2 environments (with MCM and without) × 7 bottles. One bottle per treatment (ingredient × environment) was stopped after 8 h of fermentation, and three bottles per treatment after 24 and 72 h of fermentation.

When stopped, fermentation broth and three MCMs per bottle, when applicable, were used for counting of *Lactobacillus* sp. A sample of fermentation broth was also centrifuged (12 000 *g*, 5 min) and the supernatant used for analysis of short-chain fatty acids (SCFAs), while the pellet was further used for extraction of microbial genomic DNA. In the bottles containing MCM, the mucus from the three remaining MCMs was also used for genomic DNA extraction.

Cumulative gas production

Cumulative gas pressure measurements were converted in volume, with corrections for control bottles, using the law of Boyle–Mariotte and fermentation kinetics modelled according to Groot *et al.* (1996):

$$G_i = \frac{A \times t_i^C}{t_i^C + B^C}$$

where G_i ($ml.g^{-1}$) denotes the total cumulative gas produced by one gram of organic matter weighed into the bottle, t_i (h) the incubation time, A ($ml.g^{-1}$) the asymptotic gas production, B (h) the mid-fermentation time and C the switching sigmoidal characteristics of the curve.

The maximum rate of gas production when the microbial population no longer limits the fermentation (R_{max}, $ml.h^{-1}.g^{-1}$) and the time at which the R_{max} is reached (T_{max}, h) were

Table 1. Sequences and references of the primers and probes used for performing real time PCR.

	Primers and probes	Sequence (5′–3′)	Cycles	Reference
Bifidobacterium	Forward	5′-CGCGTCYGGTGTGAAAG-3′′	45	Delroisse *et al.* (2008)
	Reverse	5′-CCCCACATCCAGCATCCA-3′		
	Probe	5′(FAM)-AACAGGATTAGATACCC-(MGB)3		
Lactobacillus	Forward	5′-GAGGCAGCAGTAGGGAATCTTC-3′	45	Delroisse *et al.* (2008)
	Reverse	5′-GGCCAGTTACTACCTCTATCCTTCTTC-3′		
	Probe	5′(VIC)-ATGGAGCAACGCCGC-(MGB)3′		
Bacteroides	Forward	5′-GAGAGGAAGGTCCCCCAC-3′	50	Layton *et al.* (2006)
	Reverse	5′-CGCTACTTGGCTGGTTCAG-3′		
	Probe	5′(VIC)-CCATTGACCAATATTCCTCACTG CTGCCT-(TAMRA)3′		
Clostridium Cluster I	Forward	5′- TACCHRAGGAGGAAGCCAC-3′	45	Song, Liu and Finegold (2004)
	Reverse	5′- GTTCTTCCTAATCTCTACGCAT-3′		
	Probe	5′-GTGCCAGCAGCCGCGGTAATACG-3′		

calculated from the second derivatives of the model's equation:

$$R_{max} = \frac{A \times B^C \times C \times T_{max}{}^{(-C-1)}}{\left(1 + B^C \times T_{max}{}^{-C}\right)^2}$$

$$T_{max} = B\left(\frac{C-1}{C+1}\right)^{\frac{1}{C}}$$

Analysis of short-chain fatty acids

Broth samples were analysed for SCFA content using a Waters 2690 HPLC system (Waters, Milford, MA, USA) fitted with an HPX 87H column (Bio-Rad, Hercules, CA, USA) combined with a UV detector (210 nm, Waters) and corrected for control values (without ingredient, with or without MCM, as appropriate). Branched-chain fatty acids (BCFAs) were calculated as the sum of *i*-butyrate, *n*-valerate, and *i*-valerate and total SCFAs were calculated as the sum of acetate, propionate, *n*-butyrate and BCFAs.

Microbial community determination

Three complementary methods were used to characterize microbial communities: *Lactobacillus* plate counts, quantitative real-time PCR (qPCR), and deep sequencing. Plate counts were used to check for viable lactobacilli cells, which contribute significantly to intestinal health by producing lactate and enhancing the gut barrier. Pyrosequencing was used to investigate potentially any bacteria (phylum, genus or species) that can be influenced by the presence of the mucus or that can grow in the mucosal layers. Finally, qPCR was run on a larger number of samples, which allowed checking for a time effect ($n = 3$) when pyrosequecing data could not be replicated for the same treatment (time × ingredient × mucus) ($n = 1$).

Lactobacillus plate counts

Fermentation broth and the mucus of three MCMs per bottle were used for counting of *Lactobacillus* sp. in colony forming units (cfu) on MRS plates (Biokar Diagnostics, Beauvais, France) after a series of 10-fold dilutions in peptone water. Each dilution was replicated three times and the plates were incubated at 37°C in an aerobic incubator with 10% CO_2 for 72 h. Only plates containing 10–300 colonies were enumerated.

DNA extraction

Genomic DNA of each fermentation broth sample was extracted using a QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions preceded by a bead beating step (FastPrep-24, MP Biomedicals, Illkirsh, France). DNA was extracted from the mucus–agar matrix of the MCM following a similar procedure with the addition of a prior enzymatic digestion of the agar. Briefly, 2 ml of the B agarase I reaction buffer (Bioké, Leiden, The Netherlands) was mixed with the three MCMs from the same bottle. The tube was placed on ice for 30 min and then incubated at 65°C for 10 min until complete dissolution of the mucus. Meanwhile the tube was regularly vortexed. The tube was then incubated at 42°C for 15 min and 5 units of B agarase I (Bioké, Leiden, The Netherlands) was added for an additional 10 min incubation at 42°C. The enzymatic digestion was finally stopped by raising the temperature to 95°C for 2 min. The tube was then centrifuged (3200 *g*, 5 min) and the pellet used to perform the DNA extraction as described above. DNA concentration and purity in extracted samples were measured by optical density using a NanoDrop ND-1000 spectrophotometer (Isogen, Sint-Pieters-Leeuw, Belgium).

Real-time PCR analysis for bacterial communities

Quantitative real-time PCR (qPCR) was performed on DNA samples to quantify four genera, *Lactobacillus*, *Bifidobacterium*, *Bacteroides* and *Clostridium* Cluster I, using the primers and TaqMan probes sequences as well as cycles listed in Table 1. The amplification and detection were carried out using a StepOnePlus sequence detection system (Applied Biosystems, Halle, Belgium) according to the authors (see references in Table 1). Standard curves were made from triplicate reads using series of 10-fold dilutions of genomic DNA extracted from colony forming unit-counted pure cultures of *Bifidobacterium longum* (LMG 13196), *Lactobacillus acidophilus* (LMG 13550), *Bacteroides fragilis* (LMG 10263) or *Clostridium perfringens* (LMG 11264). DNA extraction on these pure bacterial cultures was performed with the same method described for the fermentation broth.

Deep sequencing analyses

Deep sequencing analyses were performed on the mucus agar collected on the MCM and the fermentation broth of one

bottle per treatment ([2 ingredients + control] × 3 time points × 2 environments [with or without MCM]) and the initial inoculum.

For each sample, PCR-amplified 16S rRNA gene libraries specific for bacteria were generated with the primers E9–29 and E514–430 (Baker, Smith and Cowan 2003) selected for their theoretical ability to generate the least bias of amplification capability among the various bacterial phyla (Wang *et al.* 2013). The oligonucleotide design included A or B sequencing titanium adapters (454 Life Sciences, Roche Diagnostics, Vilvoorde, Belgium) and multiplex identifiers (MIDs) fused to the 5′ end of each primer. The amplification mix contained 5 U of FastStart high fidelity polymerase (Roche Diagnostics), enzyme reaction buffer, 200 μM dNTPs (Eurogentec, Liège, Belgium), 0.2 μM of each primer and 100 ng of genomic DNA in a volume of 100 μl. Thermocycling conditions consisted of a denaturation at 94°C for 15 min followed by 25 cycles of 94°C for 40 s, 56°C for 40 s, 72°C for 1 min and a final elongation step of 7 min at 72°C. These amplifications were performed on an Ep Master system gradient apparatus (Eppendorf, Hamburg, Germany). The PCR products were run on 1% agarose gel electrophoresis and the DNA fragments were plugged out and purified using the SV PCR purification kit (Promega Benelux, Leiden, The Netherlands). The quality and quantity of the products were assessed with a Picogreen dsDNA quantitation assay (Isogen, St-Pieters-Leeuw, Belgium).

All libraries were run in the same titanium pyrosequencing reaction using Roche MIDs. All amplicons were sequenced using the Roche GS-Junior Genome Sequencer instrument (Roche, Vilvoorde, Belgium).

The 16S rRNA gene sequence reads were processed with the MOTHUR package (Schloss *et al.* 2009). The quality of all sequence reads were de-noised using the Pyronoise algorithm implemented in MOTHUR and filtered with the following criteria: minimal length of 425 bp, an exact match to the barcode and 1 mismatch allowed to the proximal primer. The sequences were checked for the presence of chimeric amplifications using ChimeraSlayer developed by the Broad Institute (http://microbiomeutil.sourceforge.net/#A_CS) (Su *et al.* 2014).

The resulting read sets were compared with a reference dataset of aligned sequences of the corresponding region derived from the SILVA database 1.15 of full-length rRNA gene sequences (http://www.arb-silva.de/) implemented in MOTHUR (Pruesse *et al.* 2007). The final reads were clustered into operational taxonomic units (OTUs) using the nearest neighbor algorithm using MOTHUR with a 0.03 distance unit cutoff. A taxonomic identity was attributed to each OTU by comparison with the SILVA database (80% homogeneity cutoff).

As MOTHUR is not dedicated to taxonomic assignment beyond the genus level, all unique sequences for each OTU were compared with the SILVA dataset 1.15 using the BLASTN algorithm (Altschul *et al.* 1990). For each OTU, a consensus detailed taxonomic identification has been given based upon the identity (less than 1% of mismatch with the aligned sequence) and the metadata associated with the best hit (validated bacterial species or not).

Subsampled datasets were obtained and used to evaluate richness and microbial diversity using MOTHUR. Rarefaction curves (Colwell and Coddington 1994), microbial biodiversity (non-parametric (NP) Shannon diversity index; Chao and Shen 2003) and richness estimation (ACE and Chao1 estimator; Chao and Bunge 2002) were calculated. The NP Shannon index gives an estimated index value for diversity and is used when undetected species are present in the sample. The ACE and Chao1 estimators are used to estimate the real species (OTUs in this case) richness in the sample. Their values can thus be compared with the actual number of OTUs observed in samples. The ACE index differs from Chao1 as it takes into account the relative abundance of OTUs. The sequences were deposited in Genbank (no. PRJNA238296).

Statistical analyses

Gas production, SCFA production, plate counts and qPCR data were analysed for homogeneity of variances with a Bartlett test. Where required, data were log transformed. The influence of the ingredient (cellulose, inulin), the presence of MCM or not, time of incubation and their interactions on the observations were assessed using a two-way ANOVA model in the R 3.1.0 software. The Student–Newman–Keuls *post hoc* test was applied for multiple comparisons of means.

Deep sequencing results were compared to highlight differences in bacterial population between pairs of samples using a two-way ANOVA considering eta-squared for effect size and the Tukey–Kramer method for *post hoc* tests (Parks and Beiko 2010). The STAMP software was also used for paired analysis, a two-sided Fisher's exact test was performed, and confidence intervals calculated using the Newcombe–Wilson method (Altenburger *et al.* 2002). Principal component analyses (PCAs) were performed using the FactoMineR package in R 3.1.0 to determine how the different parameters, i.e. gas production, metabolites and bacterial profiles measured at genus level according to the three methods used to characterize microbial communities, clustered according to the investigated variables: the fermented ingredient (cellulose vs inulin) and the medium (broth vs MCM or broth in the presence of MCM vs broth without MCM).

RESULTS

Cumulative gas production kinetics

As shown in Table 2 and Fig. 1A and B, inulin with MCM induced higher final gas production ($A = 297$ ml.g^{-1}) and maximum rate of gas production ($R_{max} = 57.5$ ml.h^{-1}.g^{-1}) as well as faster fermentation with earlier B (5.63 h) and T_{max} (4.9 h) compared with cellulose with MCM ($A = 181$ ml.g^{-1}, $R_{max} = 10.5$ ml.h^{-1}.g^{-1}, $B = 22.52$ h, $T_{max} = 20.7$ h). Similar findings without MCM were observed, indicating that the role of the nutrient is more important than the role of MCM. Interestingly, MCM significantly increased A, and decreased B and T_{max} in inulin fermenting bottles ($P = 0.010$) while R_{max} remained unchanged ($P = 0.878$).

Short-chain fatty acid production

The control bottles (without substrate) used for inoculum correction showed significantly higher gas and SCFA production when MCM was present compared with plain bottles (data not shown). The addition of MCM did not affect total SCFA production ($P = 0.128$) (Table 3 and Fig. 1A and B). Molar ratios were modified by the presence of MCM in the fermentation bottles. The proportion of propionate increased for inulin after 24 h (41% with MCM vs 33.3% without MCM) and 72 h (39.3% with MCM vs 33% without MCM). However, due to the presence of MCM, the proportion of acetate increased for cellulose but not for inulin. The presence of MCM did not influence the molar ratio of butyrate ($P = 0.680$), except for cellulose at 24 h (6.8% with MCM vs 5.3% without MCM). The introduction of MCM reduced BCFA molar ratio when both ingredients were fermented ($P<0.001$). Lactic acid production was almost zero (data not shown).

Table 2. Gas production kinetics parameters modeled according to Groot *et al.* (1996) of the fermentation by porcine feces of celullose and inulin in the presence or absence of mucin-coated microcosms (MCM) ($n = 3$). *A*, asymptotic gas production; *B*, mid-fermentation time; R_{max}, maximum rate of gas production; T_{max}, time at which the R_{max} is reached. Data are means; values with different superscripts within a column differ significantly ($P < 0.05$).

Ingredient	MCM	*A* (ml.g^{-1})	*B* (h)	R_{max} (ml.h^{-1}.g^{-1})	T_{max} (h)
Cellulose	Without	163^{3}	22.53^{1}	9.8^{1}	20.9^{1}
	With	181^{3}	22.52^{1}	10.5^{1}	20.7^{1}
Inulin	Without	264^{2}	7.69^{2}	56.4^{2}	7.3^{2}
	With	297^{1}	5.63^{3}	57.5^{2}	4.9^{3}
SEM[a]		17	2.41	7.2	2.2
P-values					
Ingredient		<0.001	<0.001	<0.001	<0.001
MCM		0.010	0.012	0.878	<0.001
Ingredient × MCM		0.333	0.013	0.983	<0.001

[a]SEM, standard error of the mean.

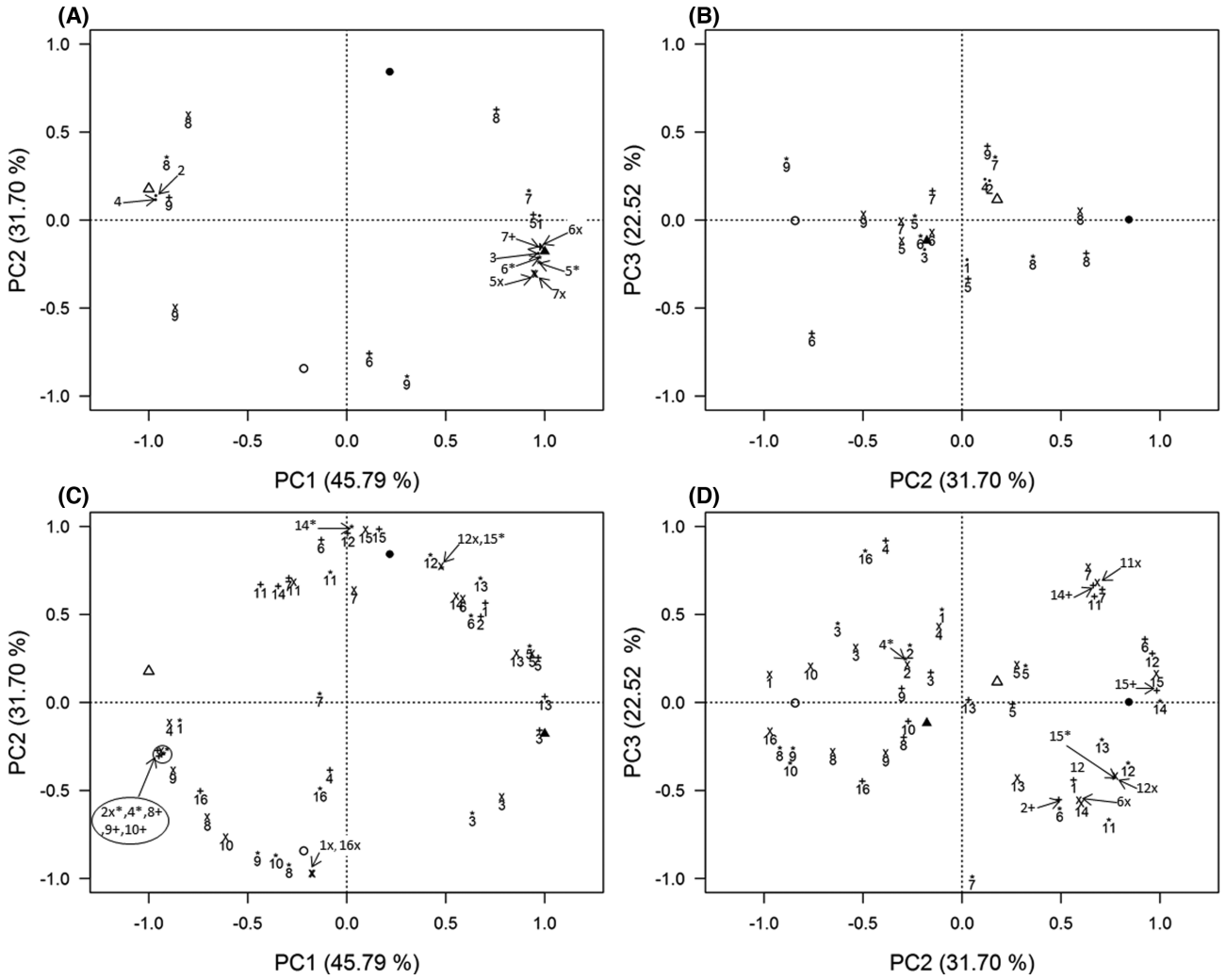

Figure 1. Principal component analysis of the quantitative measurements in the fermentation broth. The figure combines a score plot (open triangle: cellulose; closed triangle: inulin; closed circle: fermentation broth coming from bottles containing MCM; open circle: fermentation broth coming from bottles devoid of MCM) and loading plot (numbers correspond to the measured parameters). (**A**) and (**B**) show gas production and metabolites on PC1-PC2 (A) and PC2-PC3 (B); 1–9: 1 = A; 2 = B; 3 = R_{max}; 4 = T_{max}; 5 = SCFAs; 6 = acetate; 7 = butyrate; 8 = propionate; 9 = BCFAs. (**C**) and (**D**) show bacterial communities on PC1-PC2 (C) and PC2-PC3 (D); 1–16: 1 = *Lactobacillus* by plate counting; 2 = *Lactobacillus* by qPCR; 3 = *Bifidobacterium* by qPCR; 4 = *Clostridium* by qPCR; 5 = *Bacteroides* by qPCR; 6 = *Subdoligranulum* by deep sequencing; 7 = *Faecalibacterium* by deep sequencing; 8 = *Peptostreptococcaceae*_Incertae_Sedis by deep sequencing; 9 = *Turicibacter* by deep sequencing; 10 = *Clostridium* by deep sequencing; 11 = *Mogibacterium* by deep sequencing; 12 = *Bacteroides* by deep sequencing; 13 = *Parabacteroides* by deep sequencing; 14 = *Escherichia* by deep sequencing; 15 = *Akkermansia* by deep sequencing; 16 = *Corynebacterium* by deep sequencing). +: 8 h of fermentation; ×: 24 h of fermentation; *: 72 h of fermentation. For all parameters, units used to compute the PCA were consistent with data presented in the tables and figures.

Table 3. Total short-chain fatty acid (SCFA) production and molar ratios yielded after 24 and 72 h of fermentation by porcine feces of cellulose and inulin in the presence or absence of mucin-coated microcosms (MCM) ($n = 3$). Data are means; values with different superscripts within a column differ significantly ($P < 0.05$).

Incubation time (h)	Ingredient	MCM	SCFA[a] (mg.g^{-1})	Acetate (%)	Propionate (%)	Butyrate (%)	BCFA[b](%)
24	Cellulose	Without	289.4^{4}	28.3^{5}	48.2^{1,2}	5.3^{3}	18.2^{1}
		With	269.0^{4}	34.3^{4}	50.6^{1}	6.8^{2}	11.0^{2}
	Inulin	Without	670.0^{1,2}	43.9^{1}	33.3^{4}	12.6^{1}	10.3^{2}
		With	617.2^{2}	43.8^{1}	41.0^{3}	11.0^{1}	4.7^{3}
72	Cellulose	Without	518.5^{3}	37.9^{3}	48.4^{1,2}	7.3^{2}	6.3^{3}
		With	546.3^{3}	40.1^{2}	46.9^{2}	8.0^{2}	4.7^{3}
	Inulin	Without	727.6^{1}	44.3^{1}	33.0^{4}	12.8^{1}	9.9^{2}
		With	688.8^{1}	44.0^{1}	39.3^{3}	12.2^{1}	4.7^{3}
SEM[c]			34.8	1.3	1.5	0.6	0.9
P-values							
Ingredient			<0.001	<0.001	<0.001	<0.001	<0.001
MCM			0.128	0.005	<0.001	0.680	<0.001
Time			<0.001	<0.001	0.073	0.001	<0.001
Ingredient × MCM			0.078	<0.001	<0.001	0.002	0.424
Ingredient × Time			<0.001	<0.001	0.783	0.130	<0.001
MCM × Time			0.254	0.209	0.057	0.757	0.027
Ingredient × MCM × Time			0.524	0.147	0.340	0.104	0.046

[a]SCFA, short-chain fatty acid produced per gram of organic matter weighed into the bottle. [b]BCFA, branched-chain fatty acid produced per gram of organic matter weighed into the bottle. [c]SEM, standard error of the mean.

Table 4. *Lactobacillus* plate counts in fermentation broth ($n = 3$) and on mucin-coated microcosms (MCM) ($n = 3$). Data are means; values with different superscripts within a column differ significantly ($P < 0.05$); NA, not applicable.

Ingredient	MCM	Incubation time (h)	Broth *Lactobacillus*[a] (log cfu.ml^{-1})[c]	MCM *Lactobacillus*[b] (log cfu by bottle)
Control	Without	24	7.25^{1}	
		72	6.81^{2}	
	With	24	7.11^{1}	7.13^{1,2}
		72	7.02^{1,2}	6.28^{2}
Cellulose	Without	24	7.20^{1}	
		72	6.86^{2}	
	With	24	7.17^{1}	7.14^{1,2}
		72	6.86^{2}	6.16^{2}
Inuline	Without	24	7.21^{1}	
		72	6.62^{3}	
	With	24	7.17^{1}	7.53^{1}
		72	6.36^{4}	6.09^{2}
SEM[d]			0.05	
P-values				
Ingredient			<0.001	0.804
MCM			0.204	NA
Time			<0.001	<0.001
Ingredient × MCM			0.131	NA
Ingredient × Time			<0.001	0.503
MCM × Time			0.423	NA
Ingredient × MCM × Time			0.021	NA

[a]Broth *Lactobacillus*: *Lactobacillus* counted in fermentation broth. [b]MCM *Lactobacillus*: *Lactobacillus* counted on 6 MCMs in bottle. [c]cfu, colony-forming unit. [d]SEM, standard error of the mean.

Microbiota quantification by qPCR and *Lactobacillus* plate counts

Population counts decreased with time, especially between 24 and 72 h (Tables 4 and 5), but this impact of time varied with the ingredient as shown by the significant ingredient × time interactions. Although *Lactobacillus* sp. population measured via plate counts in the fermentation broth did not show any difference ascribable to the presence of MCM whatever the sampling time ($P = 0.20$) (Table 4), qPCR results showed that the addition of MCM reduced *Lactobacillus* numbers (e.g. 6.91 with MCM vs 7.10 without MCM for inulin at 72 h of fermentation) (Table 5 and Fig. 1C and D). *Bifidobacterium* populations were decreased by MCM in inulin fermenting bottles (7.44 with MCM vs 8.17 without at 24 h of fermentation), while they remained unaffected by MCM with cellulose. *Clostridium* CL I populations were not changed by the presence of MCM ($P = 0.92$) (Table 5 and Fig. 1C and D). However, *Bacteroides* increased for both ingredients (e.g 10.23 with MCM vs 10.14 without MCM for inulin at 24 h of fermentation).

Table 5. Bacterial counts in the fermentation broth ($n = 3$) and on mucin-coated microcosms (MCM) ($n = 3$) measured by qPCR. Data are means; values with different superscript numbers within a column differ significantly ($P < 0.05$); NA, not applicable.

Ingredient	MCM	Incubation time (h)	*Lactobacillus* (log cfu.ml^{-1})[a] In broth	*Lactobacillus* (log cfu.bottle^{-1}) On MCM	*Bifidobacterium* (log cfu.ml^{-1}) In broth	*Bifidobacterium* (log cfu.bottle^{-1}) On MCM	*Clostridium* (log cfu.ml^{-1}) In broth	*Clostridium* (log cfu.bottle^{-1}) On MCM	*Bacteroides* (log cfu.ml^{-1}) In broth	*Bacteroides* (log cfu.bottle^{-1}) On MCM
Control	Without	24	$7.18^{2,3}$		6.32^{5}		6.57^{2}		8.46^{10}	
		72	6.99^{4}		6.26^{5}		$6.77^{1,2}$		8.10^{11}	
	With	24	$7.21^{2,3}$		6.71^{4}		$6.72^{1,2}$		9.53^{6}	
		72	6.93^{4}	5.94	$6.52^{4,5}$	5.69	6.93^{1}	6.51	9.19^{8}	8.71
Cellulose	Without	24	7.38^{1}		7.03^{3}		6.91^{1}		9.33^{7}	
		72	6.75^{5}		6.33^{5}		6.98^{1}		8.93^{9}	
	With	24	7.25^{2}		7.11^{3}		6.91^{1}		9.90^{3}	
		72	6.64^{6}	5.72^{1}	6.39^{5}	5.67^{1}	6.92^{1}	6.59^{1}	9.48^{6}	8.92^{2}
Inulin	Without	24	7.10^{3}		8.17^{1}		$6.79^{1,2}$		10.14^{2}	
		72	6.45^{7}		7.16^{3}		$6.85^{1,2}$		9.66^{5}	
	With	24	6.91^{4}		7.44^{2}		$6.68^{1,2}$		10.23^{1}	
		72	6.17^{8}	5.41^{2}	$6.46^{4,5}$	5.76^{1}	$6.75^{1,2}$	6.09^{2}	9.77^{4}	9.03^{1}
SEM[b]			0.06	0.20	0.10	0.12	0.03	0.32	0.11	0.11
P-values										
Ingredient			<0.001	0.02	<0.001	0.28	<0.001	0.03	<0.001	0.003
MCM			<0.001	NA	0.030	NA	0.915	NA	<0.001	NA
Time			<0.001	NA	<0.001	NA	0.012	NA	<0.001	NA
Ingredient × MCM			<0.001	NA	<0.001	NA	0.027	NA	<0.001	NA
Ingredient × Time			<0.001	NA	<0.001	NA	0.173	NA	0.008	NA
MCM × Time			0.214	NA	0.643	NA	0.863	NA	0.835	NA
Ingredient × MCM × Time			0.369	NA	0.804	NA	0.905	NA	0.741	NA

[a]cfu, colony-forming unit. [b]SEM, standard error of the mean.

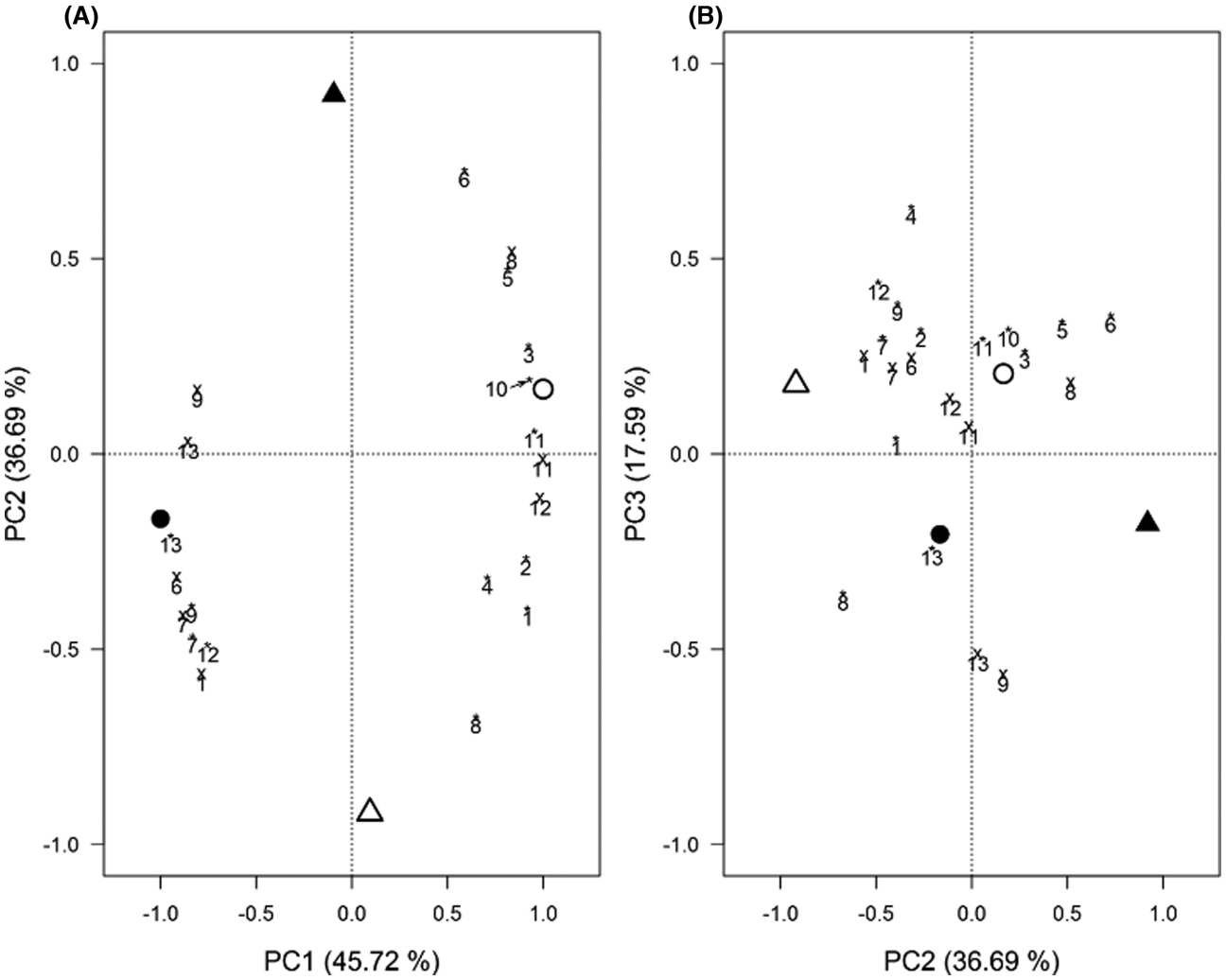

Figure 2. Principal component analysis of the bacterial measurements in the fermentation broth and on mucin-coated microcosms (MCMs). The figure combines a score plot (open triangle: cellulose; closed triangle: inulin; closed circle: MCM; open circle: fermentation broth) and loading plot (1–13 correspond the measured bacterial communities) on PC1-PC2 (A) and PC2-PC3 (B). 1 = *Lactobacillus* by plate counting; 2 = *Lactobacillus* by qPCR; 3 = *Bifidobacterium* by qPCR; 4 = *Clostridium* by qPCR; 5 = *Bacteroides* by qPCR; 6 = *Oscillibacter* by deep sequencing; 7 = *Roseburia* by deep sequencing; 8 = *Streptococcus* by deep sequencing; 9 = *Schwartzia* by deep sequencing, 10 = *Anaeroplasma* by deep sequencing; 11 = *Rikenellaceae* – RC9 by deep sequencing; 12 = *Xylanibacter* by deep sequencing; 13 = *Succinivibrio* by deep sequencing). ×: 24 h of fermentation; ∗: 72 h of fermentation. For all parameters, units used to compute the PCA were consistent with data presented in the tables and figures.

The 16S rRNA gene deep sequencing analyses highlighted further differences in microbial genera that were not included in the qPCR results (Figs 1 and 2). The presence of MCM in the bottles induced an increase in species diversity in the broth (Shannon index 5.85 vs 5.27, $P < 0.05$). As shown by the clustering along PC1, microbiota derived from broth of the MCM-containing bottles and mucus were clearly different one from the other whatever the fermented substrate (Fig. 2A), while clustering of microbial communities seemed to be influenced by both the presence of MCM and the substrate for samples from broth when incubated in the presence of MCM or not (Fig. 1C). Members of the Firmicutes phylums had a declining trend with MCM (65.27 vs 79.46%); Verrucomicrobia (5.25 vs 0.03%, $P = 0.032$) and Proteobacteria (0.55 vs 0.25%, $P = 0.013$) increased with the presence of MCM (Fig. 3).

In Figs 4 and 5 only data are given relating to genera for which significant differences ($P \leq 0.05$) were observed between the populations on mucus and in the broth or between populations of the broth when fermenting in the presence or absence of MCM. The mucus induced an increase in the share of *Ruminococcaceae* and *Bacteroidaceae* and a decrease in *Clostridiaceae* (*Clostridium genus*), *Erysipelotrichaceae* (*Turicibacter* genus) and *Peptostreptococcaceae* in the broth population (Fig. 4). The proportion of *Akkermansia* genus in broth with MCM was increased compared with that in broth without MCM Fig. 1C.

Comparing both populations in the same bottle with MCM, the proportion of *Proteobacteria* was 10 times higher in the MCM as compared with the broth (5.67% vs 0.55%, $P < 0.001$) (Fig. 3), mainly because of an increase in members of the *Succinivibrio* genus (Fig. 5).

Prevotellaceae, belonging to the Bacteroidetes phylum, were proportionally less abundant in the mucus (Fig. 5) despite the previously stated not significantly lower Bacteroidetes phylum populations in the MCM (13.7 vs 22.2%, $P = 0.14$) (Fig. 3). *Lachnospiraceae* and *Succinivibrionaceae* were promoted in MCM while *Ruminococcaceae* were proportionally more represented in the

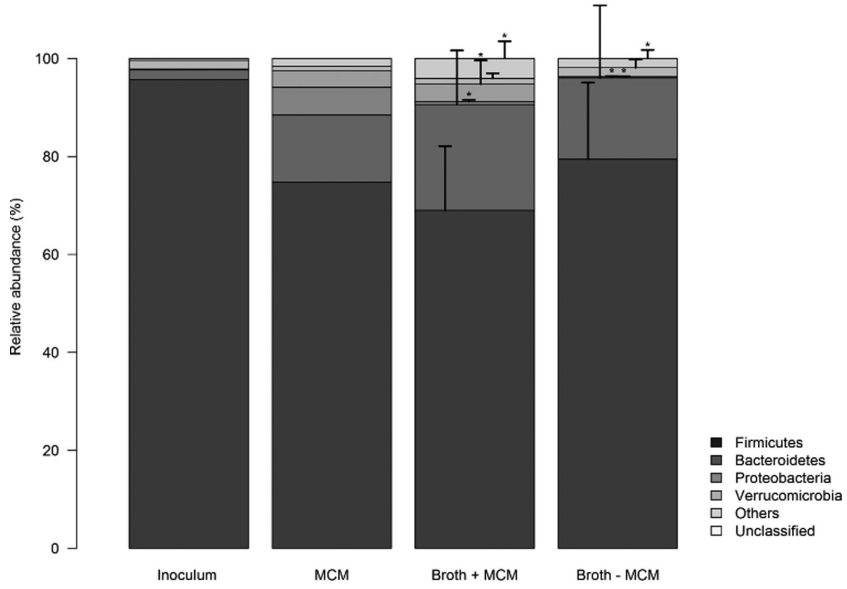

Figure 3. Share of different phyla in the bacterial population in the fermentation broths and on mucin-coated microcosms (MCM). Values presented as means ± SD (statistical analysis was performed to compare abundance of each phylum in Broth + MCM to abundance in Broth − MCM). Inoculum ($n = 1$); MCM: mucin-coated microcosms ($n = 6$); Broth + MCM: fermentation broth coming from bottles containing MCM ($n = 9$); Broth – MCM: fermentation broth coming from bottles devoid MCM ($n = 9$); Others include Actinobacteria, Fusobacteria, Spirochaetes and Synergistetes.

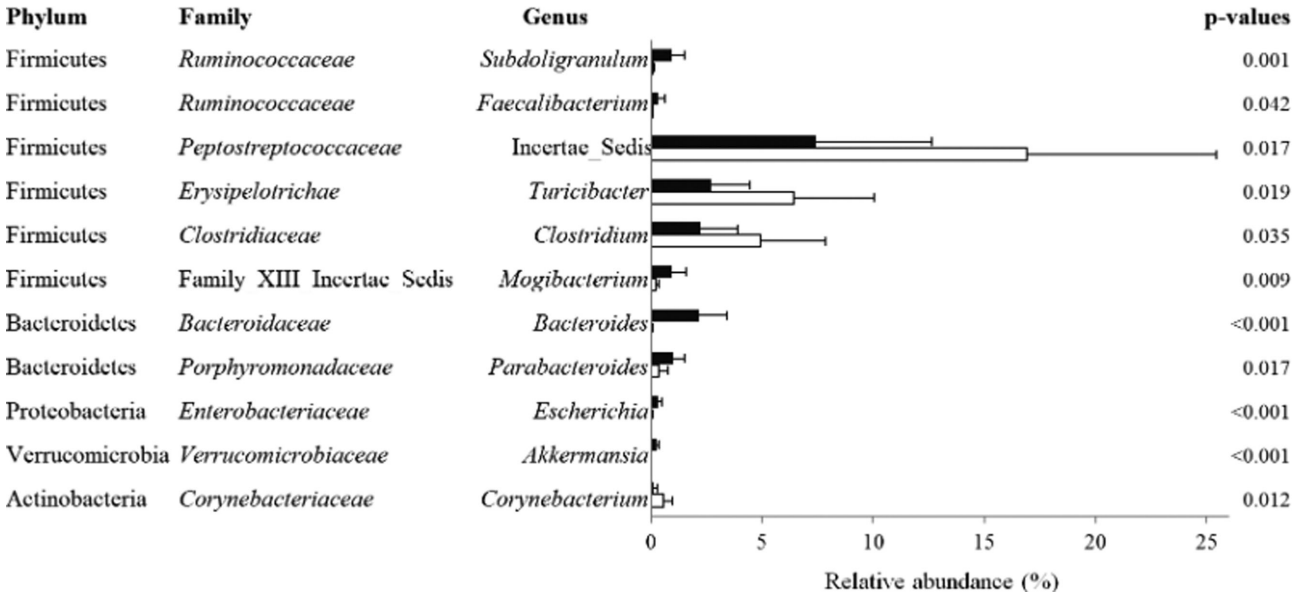

Figure 4 . Share of different genera in the bacterial communities in the fermentation broth when incubated in the presence of mucin-coated microcosms ($n = 9$) or not (MCM). Values from samples containing different ingredients (control, cellulose and inulin) at different time point (8, 24 and 72 h) were grouped to calculate the average and the SD. Broth + MCM: fermentation broth coming from bottles containing MCM (closed columns); Broth – MCM: fermentation broth coming from bottles devoid of MCM (open columns).

fermentation broth. In the same bottle, the *Lachnospiraceae* family was the most abundant and represented nearly 33% of the sequences from the MCM, against 10.4% in the fermentation broth (Fig. 5). Although not significant, differences observed for lactobacilli from the deep sequencing analyses followed the same trend as that for qPCR data, with higher populations in the broth without mucus than with mucus and a positive correlation between deep sequencing and qPCR values for lactobacilli was found ($r = 0.63$, $P < 0.029$).

Principal component analyses

PCAs were run using the whole deep sequencing dataset, but to prevent overloading of Figs 1 and 2, deep sequencing data are displayed only for the genera that are also displayed in Figs 4 and 5 since these are the ones that showed significant differences between the tested variables. As stated earlier, Fig. 2 clearly shows that the PC1, explaining 45.72% of the variability, is mostly related to differences between communities growing

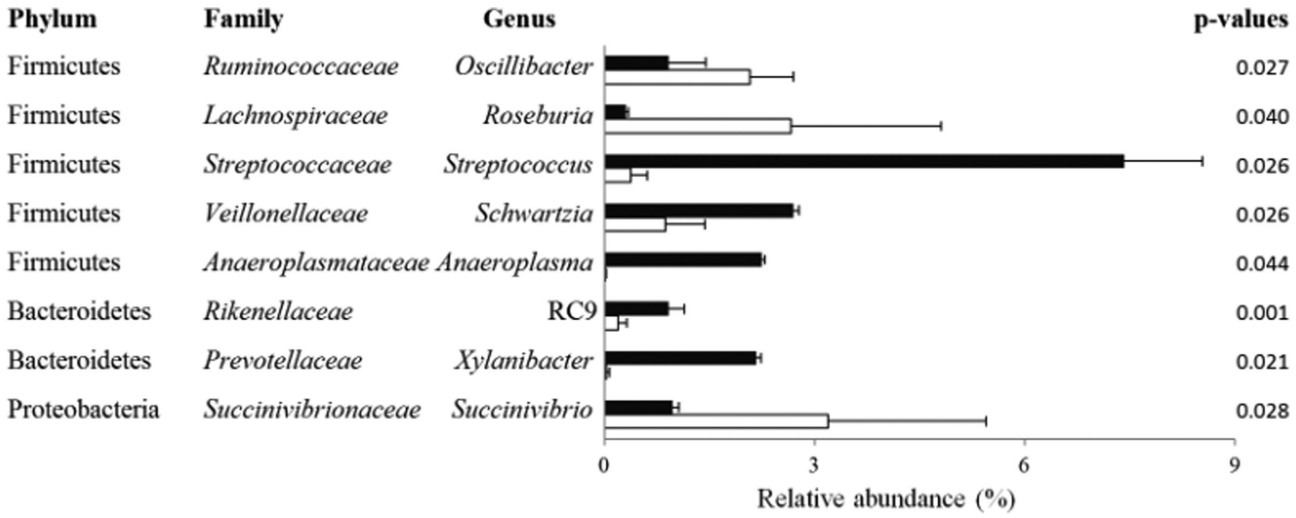

Figure 5. Share of different genera in the bacterial communities found on the mucus vs in the broth of the MCM-containing bottles ($n = 6$). Values from samples containing different ingredients (control, cellulose and inulin) at different time point (8, 24 and 72 h) were grouped to calculate the average and the SD; MCM: mucin-coated microcosms (open columns); Broth: fermentation broth of the MCM-containing bottles (closed columns).

in the broth and in the mucus. Along this axis, two very distinct bacterial communities cluster, with some correlated genera being clearly over-represented in mucus-bound communities ($r > 0.75$). Among those were found some unclassified *Lachnospiraceae*, *Oscillibacter*, *Roseburia*, *Schwartzia* and *Succinivibrio*. Figure 1 clearly shows that globally, the influence of the substrate, which was highly correlated to PC1 explaining 45.8% of the variability, was independent of that of the mucus. This axis was positively correlated to a fast ($r = 0.97$, –0.96 and –0.97 for R_{max}, B and T_{max}, respectively) and extensive fermentation ($r = 0.97$, 0.94, 0.95 and 0.97, for A and total SCFAs production after 8, 24 and 72 h, respectively) of inulin as opposed to a slower fermentation of cellulose. Molar ratios of SCFAs produced after 24 and 72 h were also highly correlated to PC1 with $r = 0.97$ and 0.99 for acetate, 0.95 and 0.92 for butyrate and –0.80 and –0.92 for propionate, after 24 and 72 h, respectively. The presence of mucus distinguished bacterial communities, with strong positive correlations ($r > 0.75$) between some specific genera and PC2 (31.7% of the variability): *Akkermansia*, *Bacteroides*, *Escherichia* and *Subdoligranulum*. During this analysis, most correlations with PC2 were true whatever the sampling time (8, 24 or 72 h).

DISCUSSION

Since mucins are heavily glycosylated proteins that are produced mainly by the epithelial cells and secreted in the gut, it is highly likely that they can be used as substrates by some bacterial species, changing the profile of bacterial populations and influencing fermentation kinetics as well as fermentation metabolites. MCMs were used to mimic the binding of microbes to the mucosal environment in order to investigate how extensively microbial communities are dependent on the presence of mucosal layers in the intestines and to see whether this dependence is substrate specific. This led us to observe some specific communities that were mucus-bound and to study the actual consequences on fermentation pathways through SCFA profiles.

Indeed, *in vitro* cultured mucins harbored a different microbiota from the fermentation broth of the bottles containing the MCM. However, surprisingly, contrary to the observations of Van den Abbeele *et al.* (2012), the presence of MCM did not favor beneficial bacteria counts such as *Lactobacillus* or *Bifidobacterium* in the broth of the *in vitro* fermentation model that was used. This discrepancy could be explained by the different system used, batch vs continuous system, where free-cell bacteria are at risk of wash-out if they do not grow fast enough. Indeed, the batch model is a closed system without any output once the fermentation has started, except for gases. Thus, bacteria can multiply and populations build up in the system over time as long as they don't die off, from substrate depletion for example. Moreover, the buffering of the solution in the batch model does not support competitiveness of lactobacilli whose domination in a population is usually ensured through an acidification of the environment by the production of lactic acid. Finally, the lack of lactic acid in the broth indicates that the lactobacilli population peaked before the first sampling, which was done after 24 h (Pieper *et al.* 2009). In pigs, after accumulation in the distal sections of the small intestine, lactate is further fermented in acetate, propionate and butyrate by *Clostridium* spp. in the hindgut (Topping and Clifton 2001). Hence, further studies using such *in vitro* batch models should consider sampling for microbiota analysis earlier than 24 h.

In vivo, mucins concentrate on the outer part of the intestinal wall, where they give the mucus its slimy structure and act as an adhesion site for *Lactobacillus* sp. (Van Tassell and Miller 2011), but also for some other bacterial species such as *Akkermansia* and members of the Bacteroidetes, especially members of the *Bacteroides fragilis* group, which rapidly colonize mucus (Macfarlane, Woodmansey and Macfarlane 2005). However, only 1% of the total colonic bacterial species (e.g. *Bacteroides* spp., *Prevotella* spp., *Clostridium* spp., *Ruminococcus* spp., *Akkermansia* spp.) are able to degrade mucus (Hoskins and Boulding 1981; Stanley *et al.* 1986; Cummings and Macfarlane 1991; Corfield *et al.* 1992; Derrien *et al.* 2004, 2010). Therefore, the presence of MCM in the system increased their concentration in the broth of MCM-containing bottles compared with the broth of bottles without MCM. Other mucin degrading bacteria such as *Akkermansia muciniphila* (Derrien *et al.* 2010; Belzer and de Vos 2012) were also enriched in the broth due to the mucus. The enrichment of

Bacteroides in the broth is probably ascribable to the competition with lactobacilli, which have to move from broth fermentation to mucus. In the present experiment, Bacteroidetes represented only 14% of all bacteria detected on the MCM, whereas Firmicutes represented 75% on the MCM. These results are close to observations in the interfold mucosal region of the mouse ascending colon (16% and 78%, respectively, Bacteroidetes and Firmicutes) (Nava, Friedrichsen and Stappenbeck 2011). The enrichment of Firmicutes on the mucus compared with the fermentation broth induced a lower ratio of Firmicutes in the broth and vice versa for the Bacteroidetes phylum. The enrichment of the *Lachnospiraceae* family belonging to the Firmicutes on the MCM suggests that these are specific groups that require adhesion to the mucus to develop in an intestinal environment. These findings are consistent with observations made *in vivo* in mice (Hill *et al.* 2010; Nava, Friedrichsen and Stappenbeck 2011; Van den Abbeele *et al.* 2013). Inside the *Roseburia* genus belonging to the *Lachnospiraceae* family, different species increased their share on the MCM, a change that was also observed *in vitro* by Van den Abbeele *et al.* (2013). In contrast, the *Ruminococcaceae* belonging to the Firmicutes phylum were lower on the MCM than in the fermentation broth, while Nava, Friedrichsen and Stappenbeck (2011) reported a higher population in this family in the interfold mucosal region than in the intestinal lumen. The proportion of *Prevotellaceae* on mucin was also lower than that in fermentation broth, consistent with the lower density in *Prevotellaceae* observed in the mucosal region compared with the digesta in mice (Nava, Friedrichsen and Stappenbeck 2011).

In addition, mucin-degrading bacteria produce extracellular glucosidases that will break down the large molecules of mucin into monosaccharides and amino acids, which can then be released in the fermentation broth and used by other less-specialized bacteria (Derrien *et al.* 2010). The presence of mucus in the *in vitro* model induces a synergetic activity that modifies the global fermentation environment also for bacteria that do not strictly depend on mucus to grow (Van den Abbeele *et al.* 2011; Belzer and de Vos 2012). In the present experiment, the production of gas and SCFAs in the control bottles containing the MCM is indeed an indication of mucin consumption as energy source by some bacteria, as described *in vivo* (Derrien *et al.* 2010). Among them, the *Ruminococcus gnavus* strains belonging to the *Lachnospiraceae* produce propionate when using mucin as carbon source (Crost *et al.* 2013), explaining the increased proportion of propionate in the presence of MCM, especially with inulin. Although, this increase in propionate molar ratio in the current *in vitro* system was 6–8%, this ratio was 2-fold higher than *in vivo*. Therefore, even a small increase in the propionate ratio can have a significant relevance for animal health. For example, a propionate concentration of 2 mM had no biological effect in human, but at 2.5 or 3 mM, a reduction in the proliferation of human and animal lymphocytes and an inhibition the production of the pro-inflammatory cytokine resistin by human adipose tissue (Al-Lahham *et al.* 2010) were observed. The investigated hypothesis of whether the presence of the MCM would shift fermentation in the broth because of a change in bacterial communities that would depend on mucus to grow was proven relevant since broth microbiota, fermentation dynamics and metabolites were modified in the presence of mucus, for both investigated substrates. The enrichment of Firmicutes on MCM might possibly have caused an increase in the BCFA ratio in the fermentation broth compared with fermentation broth without MCM. BCFAs, chiefly isobutyrate and isovalerate, which are typical products of protein fermentation, are needed for growth of several cellulolytic and non-cellulolytic bacteria (Allison *et al.* 1962). These increased Firmicutes populations could explain the decrease in absolute production of acetate with inulin, although the acetate molar ratio was not significantly lower. Indeed, Firmicutes are key players in acetate oxidation, with subsequent carbon dioxide and methane production by methanogens (Mulat *et al.* 2014) increasing gas production measurement. This degradation of acetate possibly came on top of a decrease in acetate production due to the observed reduction in populations of the acetate-producing bifidobacteria and the increase in propionate-producing Bacteroides in fermentation broth with MCM (Rios-Covian *et al.* 2013). Since the production of propionate does not lead to a concomitant production of fermentation CO_2 and CH_4, unlike acetate and butyrate, a reduction in acetate and an increase in propionate lead to an apparent uncoupling between SCFAs and gas measurement. This was observed in the case of inulin, for instance, where MCM-containing bottles had the fastest and highest gas production but did not yield significantly higher SCFA production compared with inulin without MCM and cellulose.

Regarding the second hypothesis, on the substrate specificity of the dependence of some microbial communities on the presence of mucus, most of the shifts induced by the presence of the mucus were similar for cellulose and inulin. Indeed, kinetics and total SCFA production were mostly driven by the ingredient, and the abundance of most bacterial genera depends either on the ingredient (e.g. *Clostridium* spp. *Bacteroides* spp.) or on the presence or absence of mucus irrespective of the ingredient (e.g. *Akkermansia* spp., *Coynebacterium* spp.). Indeed, as shown in Fig. 1, fermentation kinetics and microbial communities in the broth depended mostly on the fermented ingredient and, from the PCA analyses, changes that implied different linear combination between the ingredients and the presence of the mucus are noticeable in Fig. 1C and D with a much stronger weight on the effect of the mucus than the ingredient along PC2 aligned with PC3. Therefore, the presence of MCM did not influence the prebiotic properties of the ingredient. The effects of the substrates on the microbiota recovered on the MCM were limited to a small and transient increase in *Lactobacillus* and more marked decrease in *Clostridium* populations with inulin compared with cellulose, but they are in accordance with *Lactobacillus* increases and *Clostridium* decreases observed in the small and large intestinal mucosa of piglets supplemented with inulin (Patterson *et al.* 2010). Shifts in *Bifidobacterium* spp. are also in agreement with *in vivo* results in pigs (Loh *et al.* 2006; Patterson *et al.* 2010; Boudry *et al.* 2012), with a higher population of *Bifidobacterium* spp. with inulin compared with cellulose, and even the introduction of MCM in the bottles increased the *Bifidobacterium* populations in the fermentation broth with cellulose. One of the few parameters to behave differently is related to the SCFA molar ratio, especially that of propionate and to a lesser extent butyrate. Propionate increased 6–8% when mucus was added to the system with inulin, while the effect of mucus with cellulose was not significant. As stated before, this is probably a consequence of the slower fermentation of cellulose as compared with inulin (Juśkiewicz *et al.* 2009) and possibly driven by the few genera, such as acetate-producing *Bifidobacterium* spp. (Scott *et al.* 2014), which were differently influenced by the presence of mucus according to the ingredient as explained before. Finally, in the case of cellulose with or without MCM after 24 h of fermentation, the proportion of propionate was quite high (48.2% without MCM and 50.6% with MCM) compared with *in vivo* concentrations in the caecum (25%) and the colon (20%) of pigs (Boudry *et al.* 2012). The *in vitro* model seems thus less accurate for predicting

fermentation of low fermentable functional carbohydrates with an overestimation of propionate production.

CONCLUSION

It can be concluded that the inclusion of MCM in a batch fermentation model modified the fermentation kinetics and the microbiota composition, especially for populations that were found growing on mucus, even in the broth phase of the batch model that was used. Although inulin and cellulose are highly contrasted in terms of fermentation patterns, metabolites produced and microbial species that they support, the addition of MCM did not impact the bacterial differences observed between cellulose and inulin showing that when using two highly contrasted ingredients, the dependence on mucus of some bacterial genera is not influenced by the substrate. Therefore, to definitely decide whether the addition of MCM is a desirable improvement to distinguish similar CHO ingredients, a proper comparative experiment with *in vivo* data should be performed.

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Conflict of interest. None declared.

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