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Reducing agent can be omitted in the incubation medium of the batch *in vitro* fermentation model of the pig intestines

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Over the past decade, in vitro *methods have been developed to study intestinal fermentation in pigs and its influence on the digestive physiology and health. In these methods, ingredients are fermented by a bacterial inoculum diluted in a mineral buffer solution. Generally, a reducing agent such as Na_2S or cysteine-HCl generates the required anaerobic environment by releasing metabolites similar to those produced when protein is fermented, possibly inducing a dysbiosis. An experiment was conducted to study the impact of two reducing agents on results yielded by such* in vitro *fermentation models. Protein (soybean proteins, casein) and carbohydrate (potato starch, cellulose) ingredients were fermented* in vitro *by bacteria isolated from fresh feces obtained from three sows in three carbonate-based incubation media differing in reducing agent: (i) Na_2S, (ii) cysteine-HCl and (iii) control with a mere saturation with CO_2 and devoid of reducing agent. The gas production during fermentation was recorded over 72 h. Short-chain fatty acids (SCFA) production after 24 and 72 h and microbial composition of the fermentation broth after 24 h were compared between ingredients and between reducing agents. The fermentation residues after 24 h were also evaluated in terms of cytotoxicity using Caco-2 cell monolayers. Results showed that the effect of the ingredient induced higher differences than the reducing agent. Among the latter, cysteine-HCl induced the strongest differences compared with the control, whereas Na_2S was similar to the control for most parameters. For all ingredients, final gas produced per g of substrate was similar ($P > 0.10$) for the three reducing agents whereas the maximum rate of gas production (R_{max}) was reduced ($P < 0.05$) when carbohydrate ingredients were fermented with cysteine-HCl in comparison to Na_2S and the control. For all ingredients, total SCFA production was similar ($P > 0.10$) after 24 h of fermentation with Na_2S and in the control without reducing agent. Molar ratios of branched chain-fatty acids were higher ($P < 0.05$) for protein (36.5% and 9.7% for casein and soybean proteins, respectively) than for carbohydrate (<4%) ingredients. Only fermentation residues of casein showed a possible cytotoxic effect regardless of the reducing agent ($P < 0.05$). Concerning the microbial composition of the fermentation broth, most significant differences in phyla and in genera ascribable to the reducing agent were found with potato starch and casein. In conclusion, saturating the incubation media with CO_2 seems sufficient to generate a suitable anaerobic environment for intestinal microbes and the use of a reducing agent can be omitted.*

Keywords: gut fermentation, *in vitro* method, pig, reducing agent, pyrosequencing

Implications

Animal studies seek to replace *in vivo* methods with *in vitro* methods. In this study, we show that *in vitro* models, used to investigate the influence of the diet on the digestive physiology and intestinal health of pigs, can omit some specific chemicals called reducing agents to induce the oxygen-free environment required for intestinal microbes. Therefore, building on this experiment, future research using such *in vitro* models will yield results that are more relevant, helping in reducing the number of animals usually used for such studies on intestinal health.

Introduction

In vitro fermentation models are increasingly used to characterize the fermentation of fiber-rich ingredients by

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intestinal microbes and its influence on digestive physiology (e.g. Bindelle *et al*., 2011; Jha *et al*., 2015) and intestinal health of pigs (e.g. Pieper *et al*., 2009a; Cardarelli *et al*., 2016). Although *in vitro* and *in vivo* methods are not directly comparable (Weiss *et al*., 2015), Koecher *et al*. (2014) showed that both types of models provide complementary information on fermentation in the gut. In *in vitro* models, after pre-digestion by pepsin and pancreatic enzymes to simulate digestion in the stomach and in the small intestine (Bindelle *et al*., 2007; Sappok *et al*., 2009), ingredients are fermented in a closed system (syringe, flask or bottle) by a bacterial inoculum made from fresh or snap-frozen ileal, cecal or fecal material diluted in a mineral buffer solution. The required anaerobic environment is generated: (i) by carrying out feces collection and preparation of microbial inoculum under a constant stream of CO_2 or N_2 and (ii) by, except in a small number of early studies (e.g. Tilley and Terry, 1963), adding a reducing solution. Usually this solution contains Na_2S, alone (Menke and Steingass, 1988) or in combination with cysteine-HCl (Theodorou *et al*., 1994).

Such *in vitro* models lack the response of the host when exposed to the bacterial metabolites produced during the fermentation of the different ingredients, which limited their use in health related studies. To solve this limitation, *in vitro* adenocarcinoma intestinal cell culture models (Caco-2 cells or mucus-secreting HT29-MTX cell layers) have been combined to *in vitro* fermentation models (e.g. Payne *et al*., 2012).

However the addition of Na_2S and/or cysteine-HCl in the fermentation model releases metabolites such as H_2S, ammonia, thiols or amines that could be detrimental to some intestinal microbes and to the intestinal epithelium while not being present in such amounts in natural digestive physiological processes. This could induce a bias in the results from cell culture models, limiting their relevancy. H_2S, for example, is toxic to the intestinal mucosal barrier via DNA damage (Attene-Ramos *et al*., 2006), alteration of the cellular respiration (Medani *et al*., 2011) and inhibition of the butyrate oxidation in colonocytes (Roediger *et al*., 1997). Ammonia is a metabolic disruptor due to its ability to inhibit mitochondrial oxygen consumption (Andriamihaja *et al*., 2010) and short-chain fatty acids (SCFA) oxidation (Cremin *et al*., 2003) in colonic epithelial cells. Moreover, the *in vitro* microbial activity could be influenced by the reducing agent as they can precipitate essential metal ions and produce potentially toxic intermediates that may induce an imbalance between bacterial species (Fukushima *et al*., 2003). Finally, reducing agents produce molecules that are also end-products of intestinal protein fermentation, blunting the ability of *in vitro* methods to quantify the production of metabolites originating from protein fermentation. H_2S is produced by fermentation of sulfur-containing amino acids (AA) (Christl *et al*., 1992) by bacterial species commonly present in the large intestine (e.g. *Escherichia coli*, *Clostridium* spp., *Enterobacter aerogenes)* (Kumagai *et al*., 1975; Awano *et al*., 2005). Ammonia is generated in the large intestine by deamination of AA whereas the decarboxylation of AA by *Bifidobacterium*, *Clostridium, Bacteroides*, *Streptococcus*, *Lactobacillus* members can lead to the production of amines (Hughes *et al*., 2000).

In this context, the present study aimed to investigate the possibility of using an *in vitro* gas production method in combination with a model of the host's epithelium without the addition of any reducing agent in the incubation medium. For this purpose, the influence of the reducing agent (Na_2S, cysteine-HCl, or none) on kinetics of gas and SCFA production, on intestinal microbial populations and on the toxicity of fermentation residues for epithelial cells was evaluated when protein (casein or soybean proteins) and carbohydrate (cellulose or potato starch) ingredients were fermented *in vitro*.

Material and methods

Ingredients

Four ingredients were used: soybean proteins (Soycomil; ADM, Rotterdam, the Netherlands), casein (C7078; Sigma-Aldrich, St. Louis, MO, USA), potato starch (S4251; Sigma-Aldrich) and cellulose (Mikro-technik GmbH & Co. KG, Bürgstadt, Germany).

Kinetics of gas and short-chain fatty acids production during in vitro *large intestine fermentation*

In vitro *fermentation*. *In vitro* fermentation was conducted as described in Bindelle *et al*. (2007) with changes in the use of reducing agents as described hereafter. In brief, fresh feces were collected from three sows directly from the rectum and placed in plastic syringes. The air was chased from syringes and they were placed in a water-bath (39°C) for transportation to the lab. After <1 h, feces samples of the three sows were pooled in equal proportions on fresh-weight basis and added to three incubation media, prepared according to Menke and Steingass (1988) (Na_2HPO_4, 1.423 g/l; KH_2PO_4, 1.548 g/l; $MgSO_4 \times 7\ H_2O$, 0.150 g/l; $NaHCO_3$, 8.738 g/l; $(NH_4)\ HCO_3$, 0.999 g/l; $CaCl_2 \times 2\ H_2O$, 1.669 mg/l; $MnCl_2 \times 4\ H_2O$, 1.264 mg/l; $CoCl_2 \times 6\ H_2O$, 0.126 mg/l; $FeCl_3 \times 6\ H_2O$, 0.101 mg/l; resazurin, 0.129 mg/l) but differing in reducing agent: (i) Na_2S (14.3 mg/l), (ii) cysteine-HCl (25 mg/l) or (iii) control without reducing agent. The preparation was carried out under a constant stream of CO_2, and subsequently, the three media were bubbled for 30 min with CO_2.

The fermentation was initiated by mixing 200 mg of an ingredient with 30 ml of one of the three incubation media in a 140-ml glass bottle equipped with a pressure sensor module (Gas production system; Ankom Technology, Macedon, NY, USA). The experimental scheme was as follows: four ingredients (+ one blank without ingredient) × three incubation media × six replicates.

During 72 h, pressure data were regularly recorded. After 24 h, the fermentation of two replicates for each combination of ingredient × incubation medium was stopped. Fermentation broth was centrifuged (13 000 × **g**, 5 min, 4°C), the supernatant and the pellet were stored at −20°C until

further use, for SCFA analysis and for extraction of bacterial genomic DNA, respectively. Short-chain fatty acids were also analyzed after 72 h.

Gas production. Gas production curves were modeled according to Groot *et al*. (1996)

$$G = \frac{A}{1 + \frac{B^c}{t^c}}; \text{ if } t > 0,$$

where G (ml/g of dry matter (DM)) denotes cumulative gas production *v*. time; *A* (ml/g of DM), maximal gas volume for $t = \infty$; *B* (h) time at which 50% of *A* is reached; and *C*, a constant determining shape of the curve. From this equation, two other parameters were calculated: R_{max}, maximum rate of gas production (ml/g of DM × h) and T_{max}, time to reach R_{max} (h).

Measurement of short-chain fatty acids production. Fermentation supernatants were analyzed for SCFA (acetate, propionate, butyrate, isobutyrate, valerate, isovalerate) production with a Waters 2690 HPLC system (Waters, Milford, MA, USA) fitted with an Aminex HPX 87 H column (Bio-Rad Laboratories, Hercules, CA, USA) combined with an UV absorbance detector (Waters 486 tunable absorbance detector; Milford) set at 210 nm. The analysis was performed at a flow rate of 0.6 ml/min and at 40°C using 3 mM H_2SO_4 at 5% CH_3CN as eluant.

Cytotoxicity of fermentation residues

Cell culture. A Caco-2 cell line (ATCC n°HTB-37), derived from a human colon adenocarcinoma, was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were routinely cultured at 37°C in a humidified atmosphere with 5% to 10% (v/v) CO_2 in air in Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Verviers, Belgium) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Hyclone Perbio-Sciences, Erembodegem, Belgium), 1% (v/v) L-glutamine 200 mM and 1% (v/v) nonessential amino acids (NEAA) (Lonza) with weekly passage.

Preparation of fermentation supernatants. Cytotoxicity assay was performed on fermentation supernatants of the four tested ingredients and blank stopped after 24 h of fermentation. Each supernatant was filtered through a 0.22 µm filter (Millipore, Bedford, MA, USA) and diluted with 10% (v/v) of 10× concentrated DMEM (4.5 g/l glucose, 10% (v/v) FBS, 1% (v/v) L-glutamine 200 mM, 1% (v/v) NEAA (Lonza)). Five dilutions (10×, 30×, 100×, 300× and 1000×) were prepared with 1× complete DMEM from each initial supernatant mixed with 10× concentrated DMEM.

Cytotoxicity assay. Caco-2 cells were inoculated at a density of 20 000 cells/well in 96-well culture plates (Corning Costar #3596; Corning Costar Corp., Cambridge, MA, USA), and cultivated until 8 days post-confluence. Then, cells were incubated for 6 h (37°C, 5% (v/v) CO_2/air) with 100 µl of supernatants (1×, 10×, 30×, 100×, 300× and 1000× dilutions), with the culture medium alone (negative reference), or with 1% (v/v) Triton X-100 (Sigma-Aldrich) (positive reference). At the end of the incubation, the possible cytotoxic effect of fermentation supernatants was determined using a Cytotoxicity Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. This assay is based on the measurement of the activity of cytosolic lactate dehydrogenase (LDH) released in the extracellular medium of Caco-2 cells upon cell damage or necrosis. During the experiment, cell morphology was observed by phase contrast microscopy.

Determination of bacterial composition

Total bacterial DNA extraction. Centrifugation pellets collected after 24 h of fermentation were extracted for total bacterial genomic DNA using a commercial kit (QIAamp® DNA Stool Mini Kit; QIAGEN, Crawley, UK), following the manufacturer's instructions. DNA concentration and purity were measured by optical density using a NanoDrop ND-1000 (Isogen, Sint-Pieters-Leeuw, Belgium).

Bacterial 16S ribosomal RNA gene amplification and pyrosequencing. Pyrosequencing analyses were conducted as detailed in Tran *et al*. (2015), on bacterial V1 to V3 regions of the 16S rRNA gene amplified in a Ep Master system gradient apparatus (Eppendorf, Hamburg, Germany) and sequenced in the same titanium pyrosequencing reaction using the Roche 454 GS Junior Genome Sequencer (Roche Diagnostics, Vilvoorde, Belgium).

Bioinformatics analysis. Data processing from amplicon sequencing was carried out with the Genome Sequencer FLX System Software Package 2.3 (Roche Diagnostics). The raw reads were processed until operational taxonomic unit (OTU) binning and taxonomical assignation with MOTHUR v1.32 (Schloss *et al*., 2009). Raw reads denoising was performed with the Pyronoise algorithm implemented in MOTHUR. The presence of chimeric sequences was checked using ChimeraSlayer developed by the Broad Institute (http://microbiomeutil.sourceforge.net/#A_CS) (Su *et al*., 2014). A taxonomical identification based on the SILVA database V1.15 (Pruesse *et al*., 2007) to the genus level was assigned to each OTU (80% homogeneity cutoff).

In the second step, all unique sequences for each OTU were compared with the SILVA data set 1.15 gene microbial database using the Basic Local Alignment Search Tool algorithm (Altschul *et al*., 1990). For each OTU, a consensus detailed taxonomic identification was given based upon the identity (<1% of mismatch with the aligned sequence) and the metadata associated with the best hit. Rarefaction curves and biodiversity parameters were obtained from subsampled datasets using MOTHUR. Chao index and inverted Simpson index were used for richness and alpha-diversity measures, respectively. Depth of coverage was estimated with Good's coverage index (Rodriguez *et al*., 2016).

Statistical analyses

The influence of the ingredient and the reducing agent on gas fermentation parameters and on total SCFA production and molar ratio after 24 and 72 h of fermentation was analyzed using the MIXED procedure of SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). Means were separated using the LSMEANS statement with a GLM based on two criteria of classification (ingredient, reducing agent) and their interaction. Cytotoxicity data as well as microbial composition (phylum and genus levels) of fermentation broth after 24 h of fermentation were analyzed similarly.

In all cases, the fermentation flask was used as experimental unit. *Post hoc* pair-wise comparisons were done using Student *t* test. Significance was judged at $P < 0.05$.

In addition, a principal component analysis (PCA) was performed using the PRINCOMP procedure of SAS 9.4 software (SAS Institute Inc.) to determine the relationships between the ingredient and the reducing agent upon gas fermentation parameters, SCFA production, cytotoxicity and microbial composition of the fermentation broth. The correlation among the variables was determined with the loading plots of the first four eigenvalues.

Results

Kinetics of gas and short-chain fatty acids production during in vitro *large intestine fermentation*

Carbohydrate and protein ingredients differed in gas production profiles. Fermentation of carbohydrates yielded higher final gas production (*A*) than proteins ($P < 0.001$; Table 1) but *A* was similar ($P > 0.10$) among the three reducing agents for each ingredient. With higher *B* and T_{max} and lower R_{max} values, cellulose and soybean proteins fermented slower than potato starch and casein. For each ingredient, fermentation patterns with Na_2S and in the control without a reducing agent were similar and differed ($P < 0.05$) from cysteine-HCl, specifically for R_{max} and T_{max}.

For all ingredients, SCFA production was similar ($P > 0.10$) after 24 and 72 h with Na_2S and in the control without reducing agent (Table 2). Cysteine-HCl yielded higher total SCFA production for some ingredients. Branched-chain fatty acids (BCFA) ratio was similar ($P > 0.05$) between incubation media for protein ingredients and higher than with carbohydrates ($P < 0.05$).

Cytotoxicity of fermentation residues

For the initial dilution, the LDH release was higher ($P < 0.001$) after exposure to fermentation supernatants of casein than to the other ingredients and were over two-fold higher that the LDH release from the negative reference, which indicated a possible cytotoxic effect (Figure 1). Increasing dilutions of supernatants did not reach the threshold of cytotoxicity. None of the reducing agents induced a different release of LDH in the culture media ($P > 0.10$) (data not shown).

Table 1 *Gas fermentation parameters modeled according to Groot* et al. *(1996) of four ingredients fermented by pig fecal bacteria in three incubation media: with Na_2S or cysteine-HCl (Cys) or control (Ctrl) without reducing agent* (n = 2)

	Casein			Soybean proteins			Cellulose			Potato starch				*P*-values		
Parameters	Cys	Na_2S	Ctrl	Cys	Na_2S	Ctrl	Cys	Na_2S	Ctrl	Cys	Na_2S	Ctrl	SEM	*I*	*R*	*I* × *R*
A	117^{c}	122^{c}	123^{c}	109^{c}	99.3^{c}	117^{c}	343^{a}	279^{ab}	305^{ab}	248^{b}	295^{ab}	283^{ab}	19.9	<0.001	0.917	0.641
B	9.10^{d}	9.73^{d}	9.73^{d}	18.8^{c}	20.6^{c}	22.3^{c}	49.6^{a}	46.3^{ab}	42.8^{b}	9.29^{d}	8.84^{d}	8.87^{d}	3.2	<0.001	0.781	0.132
C	2.46^{fg}	2.98^{ef}	2.82^{efg}	1.43^{g}	2.09^{fg}	2.22^{fg}	4.14^{de}	5.21^{bcd}	4.93^{cd}	7.50^{a}	6.22^{abc}	6.67^{ab}	0.4	<0.001	0.695	0.375
R_{max}	9.43^{cd}	10.5^{c}	10.1^{c}	3.60^{f}	3.17^{f}	3.58^{f}	6.31^{e}	8.14^{d}	8.18^{d}	50.7^{b}	53.2^{a}	54.4^{a}	4.2	<0.001	0.001	0.030
T_{max}	5.98^{de}	7.70^{cde}	7.45^{cde}	5.54^{e}	12.2^{b}	14.2^{b}	43.0^{a}	42.9^{a}	40.6^{a}	8.96^{c}	8.39^{cde}	8.48^{cd}	3.1	<0.001	0.021	0.002

A = maximal gas volume (ml/g dry matter (DM)); B = time to reach 50% of A (h); C = constant; R_{max} = maximum rate of gas production (ml/g DM × h); T_{max} = time to reach R_{max} (h); I = ingredient; R = reducing agent.
^{a,b,c,d,e,f,g} Values within a row with different superscripts differ significantly at $P < 0.05$.

Table 2 *Total short-chain fatty acids ($SCFA_{tot}$) production (mg/g dry matter (DM)) and molar ratios (%) of acetate (C_2), propionate (C_3), butyrate (C_4) and branched-chain fatty acids (BCFA) when four ingredients are fermented by pig fecal bacteria in three incubation media: with Na_2S or cysteine-HCl (Cys) or control (Ctrl) without reducing agent (n = 2)*

		Casein			Soybean proteins			Cellulose			Potato starch				*P*-values		
Parameters	Time (h)	Cys	Na_2S	Ctrl	Cys	Na_2S	Ctrl	Cys	Na_2S	Ctrl	Cys	Na_2S	Ctrl	SEM	*I*	*R*	*I* × *R*
C_2	24	40.1^{h}	41.5^{gh}	43.0^{g}	58.5^{bc}	57.0^{cde}	57.7^{bcd}	60.2^{a}	55.5^{e}	56.8^{de}	49.9^{f}	58.8^{ab}	57.7^{bcd}	1.5	<0.001	0.003	<0.001
C_3	24	10.9^{g}	10.2^{gh}	9.93^{h}	17.1^{f}	18.5^{e}	19.1^{e}	23.7^{d}	27.3^{c}	28.7^{b}	34.6^{a}	27.5^{c}	27.6^{c}	1.6	<0.001	0.007	<0.001
C_4	24	12.2^{bcd}	11.3^{cd}	11.2^{cd}	14.0^{a}	14.6^{a}	14.2^{a}	13.2^{ab}	12.3^{bc}	11.8^{bcd}	10.8^{d}	10.9^{d}	11.0^{cd}	0.4	<0.001	0.404	0.511
BCFA	24	36.8^{a}	37.0^{a}	35.8^{a}	10.4^{b}	9.85^{b}	9.01^{b}	2.93^{d}	4.83^{c}	2.60^{d}	4.73^{c}	2.83^{d}	3.71^{cd}	2.8	<0.001	0.031	0.038
$SCFA_{tot}$	24	708^{abc}	705^{bc}	701^{c}	274^{d}	226^{e}	224^{e}	104^{f}	84.6^{f}	86.3^{f}	727^{a}	719^{abc}	726^{ab}	58.2	<0.001	0.002	0.045
$SCFA_{tot}$	72	871^{a}	834^{ab}	779^{b}	548^{de}	494^{e}	484^{e}	623^{c}	629^{c}	608^{cd}	799^{ab}	804^{ab}	797^{b}	28.3	<0.001	0.007	0.503

I = ingredient; R = reducing agent.
[a,b,c,d,e,f,g,h] Values within a row with different superscripts differ significantly at $P<0.05$.

Determination of bacterial composition

The sequencing of 16S amplicons led to the analysis of 88 088 raw reads. After cleaning and chimera removal, 67 666 reads, that is, a mean of 2255 reads/sample, were binned into 13 728 OTUs with 0.03 clustering distance. Rarefaction curves are provided in the Supplementary Figure S1. The mean value of 0.99 for the Good's coverage index at the genus level (Supplementary Table S1) shows that if the sequencing effort was deep enough to capture the dominant populations of the microbiota at the genus level which were fermenting the different substrates in the presence of the different reducing agents. Regardless of the ingredient and the reducing agent, the microbiota expressed as relative proportions of each phylum in fermentation broth was dominated by the two most abundant bacterial phyla in the porcine gut microbiota: *Firmicutes* (77.6 ± 11.19%) and *Bacteroidetes* (10.6 ± 5.94%). Other phyla included *Proteobacteria* (4.2 ± 8.20%), *Spirochaetes* (0.3 ± 0.23%) and *Verrumicrobia* (0.3 ± 0.15%). *Actinobacteria*, *Fusobacteria*, *Lentisphaerae*, *Fibrobacteres, Synergistetes* each contributed to less than 0.1% of the population. Unclassified sequences accounted for 6.6 ± 3.63%.

Relevant differences were found in the relative proportions of these phyla according to the ingredient. By comparison with the fermentation blank, the proportion of *Firmicutes* was reduced for potato starch, soybean proteins and cellulose (86.34% *v.* 62.99%, 71.50%, 78.22%, respectively; $P<0.001$) whereas, *Bacteroidetes* were more abundant for potato starch and soybean proteins (6.06% *v.* 13.16%, 20.82%; $P<0.05$). Fermentation broths of potato starch presented a higher proportion of *Proteobacteria* as compared with the blanks (17.45% *v.* 0.75%; $P<0.0001$).

In addition to the ingredient, the microbial communities changed with the reducing agent. At the phylum level, the comparison of the three reducing solutions for a given ingredient (Table 3) did not reveal any difference between Na_2S and control without reducing agent ($P>0.10$). But the use of cysteine-HCl with potato starch modified the share of *Firmicutes* and *Proteobacteria*. The reduction in *Firmicutes* (47.56% *v.* 71.70%, 69.71%; $P<0.0001$) was compensated by a three-fold increase in *Proteobacteria* with cysteine-HCl compared with Na_2S or control (31.77% *v.* 10.74%, 9.83%, respectively; $P<0.0001$). When casein was fermented with cysteine-HCl, the fermentation broth contained higher proportions of *Proteobacteria* than with Na_2S or without any reducing agent (2.77% *v.* 0.33%, 0.35%; $P<0.05$).

At the genus level, only proportions of genera for which significant differences ($P<0.05$) were observed between the three reducing solutions for at least one ingredient are given in Tables 4 and 5. Similarly to the phylum level, the most significant differences in genera were found with potato starch and casein. For potato starch, several genera belonging to the *Firmicutes* (*Streptococcus*, *Blautia*, *Lachnospiraceae* unclassified, *Veillonellaceae* unclassified, *Erysipelotrichaceae* Incertae sedis) were less abundant with cysteine-HCl than with Na_2S and/or the control ($P<0.05$).

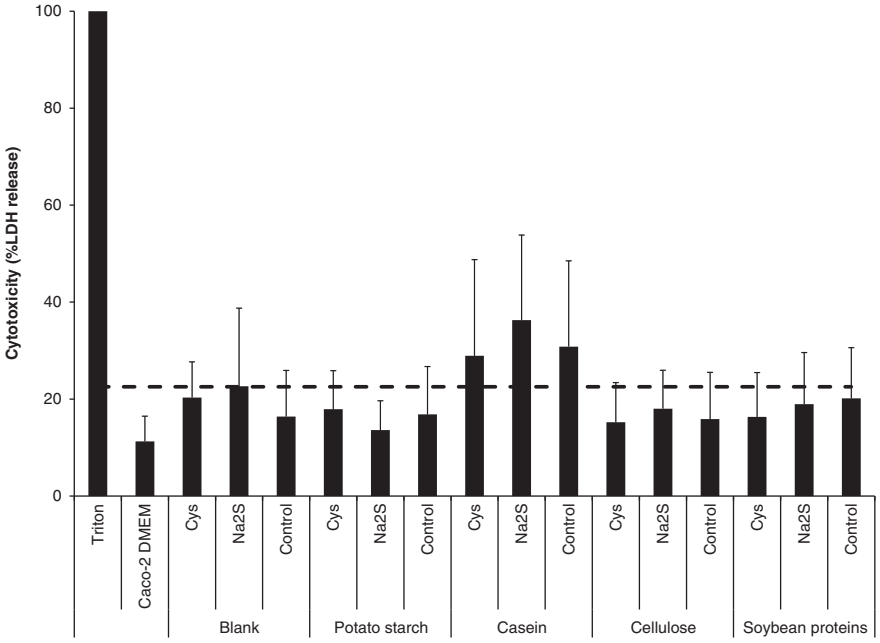

Figure 1 Cytotoxic effects on intestinal mucosa upon exposure of Caco-2 cells to fermentation supernatants (initial dilution) of the four tested ingredients and blank of fermentation in three incubation media: with Na_2S or cysteine-HCl (Cys) or control without reducing agent. Comparison with incubation medium alone (negative reference) or supplemented with 1% Triton X-100 (positive reference). The cytotoxicity was determined by assaying the lactate dehydrogenase (LDH) activity released in the culture medium and is given as means ± SD ($n = 8$). Values are representative of two independent experiments. The horizontal discontinuous line represents the threshold of toxicity.

Higher proportions of *Proteobacteria* with potato starch with cysteine-HCl than with Na_2S and control could be attributed to an increase in *Succinivibrio* (28.87% *v.* 9.08%, 8.24%; $P < 0.0001$). Within *Proteobacteria*, *Escherichia* increased with cysteine-HCl compared with Na_2S and control for the fermentation of both protein ingredients ($P < 0.01$). For casein, *Lachnospiraceae* Incertis sedis and *Dorea* were more abundant when casein was fermented with cysteine-HCl ($P < 0.01$), whereas the opposite trend was observed for *Fusobacterium* ($P < 0.0001$).

Principal component analysis of the influence of the ingredient and the reducing agent

A PCA was performed based on the correlation matrix from the complete data set (Figures 2 and 3). The first two principal components (PC1 to PC2), which explained 61.3% of the variability in the data set, clearly discriminated samples according to the ingredient. High values along PC1 reflected high degrees of cytotoxicity of casein, combined to high BCFA production that was counterbalanced by reduced acetate and propionate ratios compared with the other ingredients. PC2 mainly discriminated ingredients according to the fermentation kinetics measured through B, T_{max} and R_{max}. Similarly, PC3 was mainly influenced by the ingredient and explained 16.3% of the observed variability. High proportion of *Bacteroidetes* after 24 h for soybean proteins was translated into high negative scores along PC3. PC4, accounting for 5.8% of the variance, showed a separation according to the reducing agent for casein and potato starch. High proportions of *Fusobacterium* and *Escherichia*, as observed when casein was fermented with cysteine-HCl, were associated with low values along PC4. Similarly, potato starch fermented with cysteine-HCl showed a high proportion of *Succinivibrio*, leading to a negative value along PC4 whereas a positive value was obtained with Na_2S or the control.

Discussion

Comparing fermentation kinetics, SCFA production, cytotoxicity of the fermentation products and finally the microbiota fermenting several contrasted ingredients under three different reducing conditions (with cysteine-HCl, with Na_2S, without any reducing agent) showed that the different reducing agents accounted for a limited part of the variability. Differences in the investigated response variables that can be obtained from such *in vitro* models were by far more influenced by the ingredient than the reducing agent since the three first PCs accounted for more than 75% of the variability. This was highlighted by the low share of variability (5.8%) explained by PC4 in the PCA and the fact that clusters grouped the different ingredients whatever the reducing agent along the first three PCs. Looking more specifically at individual response variables, final fermentability of the ingredients as measured through total gas production (A) was not influenced by the reducing agent added to the fermentation broth. Nonetheless, Morgan *et al.* (2004) observed with some feed ingredients a negative impact of

Table 3 *Distribution of different phyla in the bacterial population (%) after 24 h of fermentation of the four tested ingredients and blank of fermentation by pig fecal bacteria in three incubation media: with Na_2S or cysteine-HCl (Cys) or control (Ctrl) without reducing agent (n = 2)*

	Casein			Soybean proteins			Cellulose			Potato starch			Blank			*P*-values		
Phylum	Cys	Na_2S	Ctrl	Cys	Na_2S	Ctrl	Cys	Na_2S	Ctrl	Cys	Na_2S	Ctrl	Cys	Na_2S	Ctrl	SEM	*I*	*R*	*I* × *R*
Bacteroidetes	5.39^{d}	6.03^{cd}	5.38^{d}	20.0^{a}	20.3^{a}	22.2^{a}	7.88^{c}	7.43^{cd}	7.36^{cd}	13.5^{b}	12.6^{b}	13.3^{b}	5.26^{d}	6.46^{cd}	6.46^{cd}	1.08	<0.001	0.583	0.641
Firmicutes	87.3^{a}	89.0^{a}	91.2^{a}	71.8^{cd}	72.4^{cd}	70.3^{d}	76.6^{bc}	79.2^{b}	78.8^{b}	47.5 ^{e}	71.7^{cd}	69.7^{d}	87.5^{a}	85.5^{a}	86.0^{a}	2.04	<0.001	<0.001	<0.001
Proteobacteria	2.77^{c}	0.330^{d}	0.348^{d}	2.15^{cd}	1.23^{cd}	0.958^{cd}	0.512^{d}	0.466^{d}	0.462^{d}	31.8^{a}	10.7^{b}	9.83^{b}	0.793^{cd}	0.674^{cd}	0.791^{cd}	1.49	<0.001	<0.001	<0.001
Spirochaetes	0.220^{ab}	0.179^{ab}	0.142^{b}	0.638^{a}	0.527^{ab}	0.614^{a}	0.335^{ab}	0.524^{ab}	0.287^{ab}	0.151^{b}	0.280^{ab}	0.313^{ab}	0.153^{b}	0.318^{ab}	0.313^{ab}	0.04	0.041	0.801	0.945
Verrucomicrobia	0.176^{bc}	0.286^{bc}	0.219^{bc}	0.108^{c}	0.101^{c}	0.097^{c}	0.276^{bc}	0.284^{bc}	0.183^{bc}	0.358^{ab}	0.524^{a}	0.497^{a}	0.205^{bc}	0.366^{ab}	0.364^{ab}	0.03	<0.001	0.173	0.703
Unclassified	3.23^{bc}	3.88^{bc}	2.37^{c}	5.15^{bc}	5.34^{bc}	5.66^{bc}	14.1^{a}	11.9^{a}	12.7^{a}	6.57^{b}	3.92^{bc}	6.29^{b}	5.84^{bc}	6.33^{b}	5.83^{bc}	0.66	<0.001	0.63	0.725
Others[1]	0.871^{a}	0.320^{bc}	0.366^{b}	0.203^{bcd}	0.140^{bcd}	0.117^{bcd}	0.222^{bcd}	0.189^{bcd}	0.212^{bcd}	0.0578^{d}	0.181^{bcd}	0.0701^{cd}	0.224^{bcd}	0.336^{b}	0.264^{bcd}	0.04	<0.001	0.15	0.036

I = ingredient; R = reducing agent.
^{a,b,c,d} Values within a row with different superscripts differ significantly at $P<0.05$.
[1]Others bacteria include Actinobacteria, Fusobacteria, Lentisphaerae, Synergistetes, Candidate_division_TM7, Fibrobacteres.

the use of cysteine-HCl on rumen microbes activity *in vitro*, which were reflected in this study by changes in fermentation kinetics. The addition of cysteine-HCl to the buffer solution reduced the maximum rate of gas production (R_{max}) for the carbohydrate ingredients. The second reducing agent (Na_2S) did not influence the fermentation parameters for any of the tested ingredients. Casein and potato starch fermented faster than soybean proteins and in turn, soybean proteins fermented faster than cellulose. This is explained, in the absence of any enzymatic pre-digestion of the ingredients, by differences in solubility of the nutrients and water-holding capacity of the polymers.

Although the total SCFA production was not influenced by the reducing agent for casein and potato starch, the molar ratio of propionate was higher when cysteine-HCl was used by comparison with the control devoid of reducing agent. Higher propionate was counterbalanced by a reduced ratio of acetate and is explained by the higher proportion of *Proteobacteria* for casein and potato starch fermentation with cysteine-HCl since some *Proteobacteria* are specifically involved in propionate production (e.g. *Succinivibrio dextrinosolvens*) (Watanabe *et al*., 2010).

From a phylum perspective, the microbiota, dominated by *Firmicutes* (77.6 ± 11.19%) followed by *Bacteroidetes* (10.6 ±5.94%), was in agreement with Guo *et al*. (2008) and Kim *et al*. (2011), regardless of the ingredient and the reducing agent. The domination of the bacterial communities after 24 h of fermentation by these two phyla, composed principally of anaerobes, confirms the strict anaerobic conditions in all fermentation bottles, including in the controls devoid of reducing agent. Differences in microbial communities and SCFA profiles between the three reducing conditions after 24 h were more important with ingredients for which the fermentation started earlier, namely casein and potato starch. This means that although the influence of the reducing agent is less important than that of the ingredient, it significantly affects the fermentation pathways and bacteria that are growing in the broth, especially when substrates are being metabolized intensively by the microbial communities. So for casein and potato starch, the proportion of *Proteobacteria* was considerably higher when cysteine-HCl was used as reducing agent. This was ascribed to a proliferation of the *Escherichia* and *Succinivibrio* genera for the fermentation of casein and potato starch, respectively. Moreover among the *Fusobacteria* phylum, the proportion of the *Fusobacterium* genus was increased for the fermentation of casein in the presence of cysteine-HCl in the fermentation broth. These three genera are presumed to have the capacity to degrade cysteine-HCl. Indeed, *Escherichia* are able to degrade cysteine-HCl by means of specific desulfhydrases (Awano *et al*., 2005). Gomez-Alarcon *et al*. (1982) studied the nutrient requirements of *S. dextrinosolvens* and reported that the addition of cysteine to the culture medium promotes its growth, leading to increased propionate production as mentioned before. Regarding the *Fusobacterium* genus, *F. nucleatum*, found in the oral cavity and in infected sites of healthy and sick people, was also reported as able to

Table 4 *Distribution of different genera in the bacterial population (%) after 24 h of fermentation of the four tested ingredients and blank of fermentation by pig fecal bacteria in three incubation media: with Na_2S or cysteine-HCl (Cys) or control (Ctrl) without reducing agent (n = 2)*

Phylum	Firmicutes							
Genus	*Streptococcus* spp.	*Mogibacterium* spp.	*Blautia* spp.	*Lachnospiraceae* i.s.	*Roseburia* spp.	*Dorea* spp.	*Lachnospiraceae* uncl.	*Ruminococcaceae* uncl.
Casein								
Cys	0.130^{b}	0.281^{b}	0.0553^{d}	6.12^{a}	0.000^{c}	4.12^{a}	22.4^{b}	14.3^{c}
Na_2S	0.0355^{b}	2.97^{a}	0.0484^{d}	4.71^{b}	0.000^{c}	2.47^{b}	20.6^{bc}	15.9^{abc}
Ctrl	0.0796^{b}	2.81^{a}	0.135^{cd}	4.82^{b}	0.000^{c}	2.53^{b}	19.7^{bcd}	17.6^{ab}
Soybean proteins								
Cys	0.0316^{b}	0.000^{b}	0.217^{c}	4.81^{b}	0.339^{b}	0.509^{c}	29.0^{a}	8.56^{de}
Na_2S	0.0854^{b}	0.000^{b}	0.105^{cd}	4.27^{b}	0.628^{a}	0.211^{c}	28.4^{a}	10.3^{d}
Ctrl	0.0328^{b}	0.0156^{b}	0.132^{cd}	4.06^{bc}	0.605^{a}	0.229^{c}	30.0^{a}	9.96^{d}
Cellulose								
Cys	0.121^{b}	0.0489^{b}	0.0827^{cd}	1.50^{f}	0.000^{c}	0.438^{c}	17.2^{def}	15.0^{bc}
Na_2S	0.124^{b}	0.0242^{b}	0.0644^{cd}	2.54^{de}	0.000^{c}	0.213^{c}	18.6^{cdef}	18.3^{a}
Ctrl	0.0598^{b}	0.000^{b}	0.148^{cd}	1.77^{ef}	0.000^{c}	0.198^{c}	18.0^{cdef}	16.6^{abc}
Potato starch								
Cys	0.154^{b}	0.0156^{b}	0.536^{b}	3.29^{cd}	0.0458^{c}	0.346^{c}	19.7^{bcde}	6.09^{e}
Na_2S	3.90^{a}	0.0193^{b}	1.16^{a}	3.32^{cd}	0.000^{c}	0.340^{c}	27.5^{a}	9.23^{de}
Ctrl	1.03^{b}	0.0109^{b}	1.13^{a}	3.39^{cd}	0.0416^{c}	0.0591^{c}	28.8^{a}	9.29^{de}
Blank								
Cys	0.256^{b}	0.0127^{b}	0.0641^{cd}	1.48^{f}	0.000^{c}	0.628^{c}	17.0^{def}	14.6^{bc}
Na_2S	0.0705^{b}	0.0285^{b}	0.0856^{cd}	1.62^{f}	0.000^{c}	0.112^{c}	16.6^{ef}	16.2^{abc}
Ctrl	0.125^{b}	0.0376^{b}	0.101^{cd}	1.46^{f}	0.0125^{c}	0.239^{c}	16.0^{f}	15.0^{bc}
SEM	0.192	0.186	0.0677	0.275	0.0406	0.222	0.959	0.715
P-values								
I	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
R	0.024	0.004	0.002	0.191	0.108	0.016	0.084	0.008
I × *R*	0.002	<0.001	<0.001	0.038	0.026	0.258	0.002	0.775

i.s. = incertae sedis; uncl. = unclassified; I = ingredient; R = reducing agent.
[a,b,c,d,e,f] Values within a column with different superscripts differ significantly at $P<0.05$.

degrade cysteine with specific desulfhydrases (Fukamachi *et al*., 2002).

Using culture-independent molecular techniques, some studies demonstrated that dietary changes affect the complex microbiota of the gastro-intestinal tract and lead to shifts in bacterial communities (Leser *et al*., 2000; Pieper *et al*., 2009b). So the higher differences in phyla according to the ingredient than the reducing agent are not really surprising. For example, potato starch led to higher proportions of *Proteobacteria* as compared with the other ingredients that could be attributed mainly to *Succinivibrio*, since its proliferation in the rumen was associated to a high-starch diet (O'Herrin and Kenealy, 1993). Also, *Bacteroidetes* were more abundant with potato starch and soybean proteins than in the blanks devoid of ingredients. This observation can be explained by the requirements in polysaccharides of the *Bacteroidetes.* In the human large intestine, the stability and coexistence of closely related members in this phylum was shown to be based on an synergetic interaction network related to the breakdown and utilization of dietary polysaccharides (Rakoff-Nahoum *et al*., 2014).

Results from the Caco-2 assay indicate that the reducing agent incorporated in the fermentation broth has a negligible impact on intestinal cytotoxicity of the fermentation supernatants. Despite their colorectal origin, Caco-2 cells were proven a valuable *in vitro* model of the human and pig intestinal epithelium to assess toxicity of fermentation products (Artursson *et al*., 2012). Indeed, differences in ingredients were observed comforting the relevancy of the test. The higher cytotoxicity of casein can be ascribed to its fast fermentation and by the production of toxic metabolites by the bacterial population such as ammonia, H_2S, thiols and amines, which are usually produced by protein fermenting bacteria (Hughes *et al*., 2000).

Conclusion

It can be concluded that *in vitro* intestinal fermentation models used in pig studies to investigate shifts in microbial fermentation and communities should avoid the use of cysteine-HCl as reducing agent. The addition of Na_2S seems useless if appropriate CO_2 saturation is realized and, since it probably blunts the ability to detect some

Table 5 *Distribution of different genera in the bacterial population (%) after 24 h of fermentation of the four tested ingredients and blank of fermentation by pig fecal bacteria in three incubation media: with Na_2S or cysteine-HCl (Cys) or control (Ctrl) without reducing agent (n = 2)*

Phylum	Firmicutes		Bacteroidetes		Fusobacteria	Proteobacteria	
Genus	*Veillonellaceae* uncl.	*Erysipelotrichaceae* i.s.	*Xylanibacter* spp.	RF16 uncl.	*Fusobacterium* spp.	*Succinivibrio* spp.	*Escherichia* spp.
Casein							
Cys	0.342^{cde}	0.0284^{c}	0.0284^{cd}	0.136^{bcd}	0.430^{a}	0.0134^{c}	1.96^{a}
Na_2S	0.276^{cde}	0.0129^{c}	0.0387^{cd}	0.291^{abc}	0.0258^{b}	0.0484^{c}	0.0970^{d}
Ctrl	0.189^{de}	0.0324^{c}	0.0148^{d}	0.130^{cd}	0.0176^{b}	0.0296^{c}	0.0769^{d}
Soybean proteins							
Cys	0.144^{e}	0.0136^{c}	0.443^{b}	0.126^{cd}	0.0452^{b}	1.16^{c}	0.516^{b}
Na_2S	0.158^{e}	0.000^{c}	0.498^{ab}	0.124^{cd}	0.000^{b}	0.960^{c}	0.112^{d}
Ctrl	0.183^{de}	0.0156^{c}	0.628^{a}	0.0955^{cd}	0.000^{b}	0.644^{c}	0.0830^{d}
Cellulose							
Cys	0.389^{cd}	0.0225^{c}	0.0905^{cd}	0.177^{bcd}	0.0152^{b}	0.0529^{c}	0.257^{bcd}
Na_2S	0.273^{cde}	0.0159^{c}	0.111^{cd}	0.506^{a}	0.000^{b}	0.0523^{c}	0.161^{d}
Ctrl	0.327^{cde}	0.0190^{c}	0.136^{cd}	0.243^{bc}	0.000^{b}	0.163^{c}	0.104^{d}
Potato starch							
Cys	0.483^{c}	0.106^{c}	0.513^{ab}	0.0313^{d}	0.0144^{b}	28.9^{a}	0.177^{cd}
Na_2S	1.23^{a}	1.20^{a}	0.188^{c}	0.0265^{d}	0.000^{b}	9.08^{b}	0.232^{cd}
Ctrl	1.01^{b}	0.648^{b}	0.438^{b}	0.145^{bcd}	0.000^{b}	8.24^{b}	0.138^{d}
Blank							
Cys	0.218^{de}	0.0962^{c}	0.0641^{cd}	0.224^{bc}	0.0193^{b}	0.0509^{c}	0.429^{bc}
Na_2S	0.186^{de}	0.0437^{c}	0.0875^{cd}	0.475^{a}	0.000^{b}	0.122^{c}	0.234^{cd}
Ctrl	0.251^{de}	0.0754^{c}	0.113^{cd}	0.351^{ab}	0.000^{b}	0.163^{c}	0.201^{cd}
SEM	0.0586	0.0600	0.0393	0.0294	0.0212	1.38	0.0861
P-values							
I	<0.001	<0.001	<0.001	0.001	0.002	<0.001	<0.001
R	0.071	<0.001	0.086	0.020	0.003	<0.001	<0.001
I × *R*	0.001	<0.001	0.045	0.232	0.003	<0.001	<0.001

uncl. = unclassified; i.s. = incertae sedis; I = ingredient; R = reducing agent.
[a,b,c,d,e] Values within a column with different superscripts differ significantly at $P<0.05$.

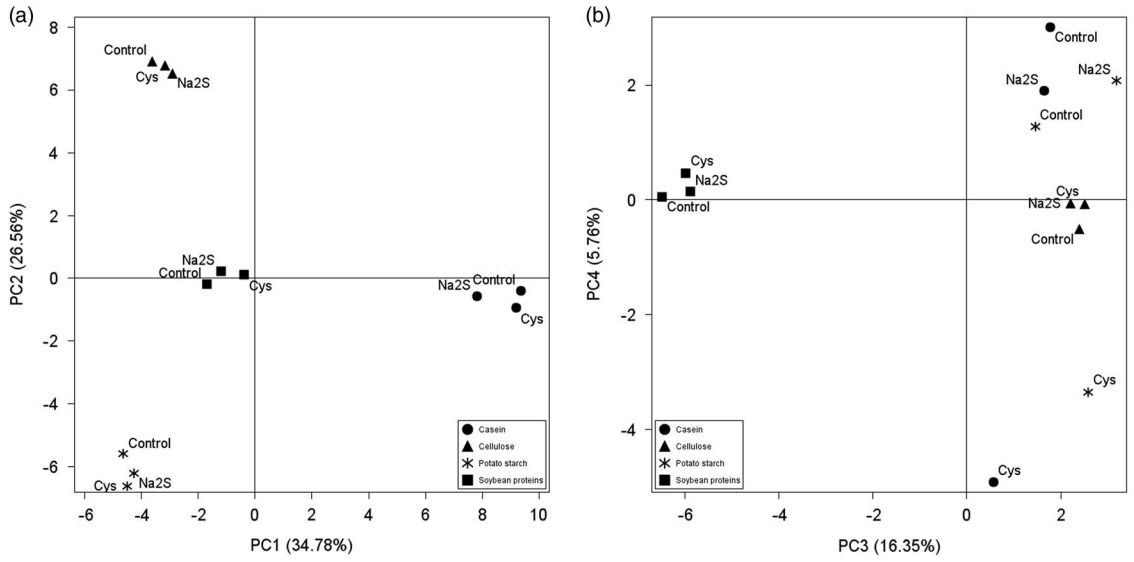

Figure 2 Score plot from the first four principal components (PC1 to PC4). Different symbols indicate the scores of the four ingredients according to the reducing agent incorporated in the fermentation broth: Na_2S, cysteine-HCl (Cys) or control without reducing agent (control). Symbols are: ● = Casein; ▲ = Cellulose; ✳ = Potato starch; ■ = Soybean proteins.

protein fermentation metabolites, it can easily be omitted. Finally, when fermentation broths are applied to Caco-2 cells culture to simulate the effect of bacterial metabolites on the intestinal epithelium, the impact of the ingredient is detectable regardless of the reducing agent used, as for most of the response variables measured in the present study.

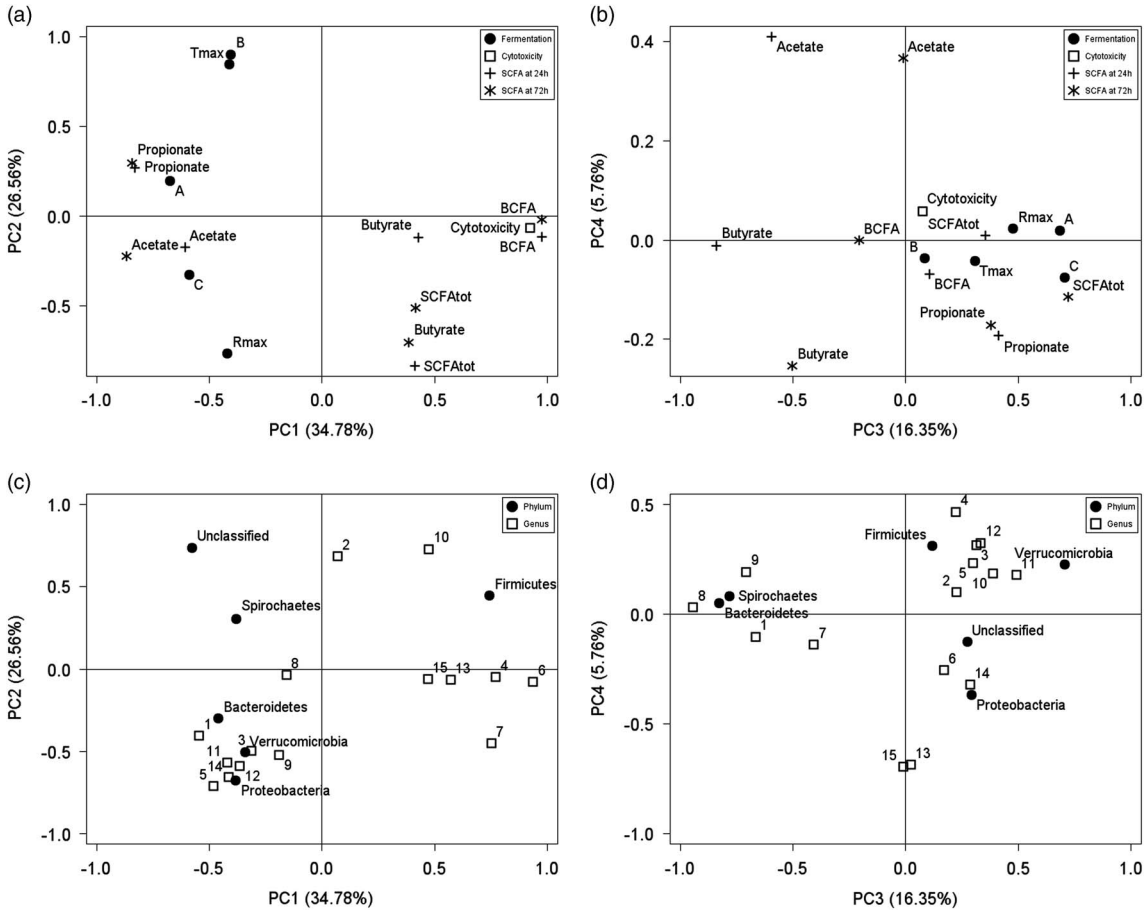

Figure 3 Loading plot from the first four principal components (PC1 to PC4) describing the relationships between fermentation parameters, short-chain fatty acids (SCFA) production after 24 and 72 h of fermentation, cytotoxicity (a and b) and microbial composition (c and d). A = maximal gas volume; B = time to reach 50% of A; C = constant; R_{max} = maximum rate of gas production; T_{max} = time to reach R_{max}; $SCFA_{tot}$ = total SCFA production; BCFA = branched-chain fatty acids; 1 = *Xylanibacter* spp.; 2 = *RF16* unclassified; 3 = *Streptococcus* spp.; 4 = *Mogibacterium* spp.; 5 = *Blautia* spp.; 6 = *Dorea* spp.; 7 = *Lachnospiraceae* Incertae Sedis; 8 = *Roseburia* spp.; 9 = *Lachnospiraceae* unclassified; 10 = *Ruminococcaceae* unclassified; 11 = *Veillonellaceae* unclassified; 12 = *Erysipelotrichaceae* Incertae Sedis; 13 = *Fusobacterium* spp.; 14 = *Succinivibrio* spp.; 15 = *Escherichia* spp.

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Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731117002749

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