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In vitro prebiotic potential of agricultural by-products on intestinal fermentation, gut barrier and inflammatory status of piglets

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Abstract

The inclusion of fibre-rich ingredients in diets is one possible strategy to enhance intestinal fermentation and positively impact gut ecology, barrier and immunity. Nowadays, inulin-type fructans are used as prebiotics in the feed of piglets to manipulate gut ecology for health purposes. Likewise, some by-products could be considered as sustainable and inexpensive ingredients to reduce gut disorders at weaning. In the present study, chicory root and pulp, citrus pulp, rye bran and soya hulls were tested in a three-step *in vitro* model of the piglet's gastro-intestinal tract combining a pepsin-pancreatin hydrolysis (digestion), a dialysis step using cellulose membranes (absorption) and a colonic batch fermentation (fermentation). The fermentation kinetics, SCFA and microbiota profiles in the fermentation broth were assessed as indicators of prebiotic activity and compared with the ones of inulin. The immunomodulatory effects of fermentation supernatant (FS) were investigated in cultured intestinal porcine epithelial cells (IPEC-J2) by high-throughput quantitative PCR. Chicory root displayed a rapid and extensive fermentation and induced the second highest butyrate ratio after inulin. Citrus pulp demonstrated high acetate ratios and induced elevated *Clostridium* clusters IV and XIVa levels. Chicory root and pulp FS promoted the intestinal barrier integrity with up-regulated tight and adherens junction gene expressions in comparison with inulin FS. Chicory pulp FS exerted anti-inflammatory effects in cultured IPEC-J2. The novel approach combining an *in vitro* fermentation model with IPEC-J2 cells highlighted that both chicory root and pulp appear to be promising ingredients and should be considered to promote intestinal health at weaning.

Key words: **By-products: *In vitro* fermentation: Intestinal porcine epithelial cells: SCFA: Microbiota**

Prebiotics are non-digestible feed ingredients selectively affecting the growth and activity of beneficial bacteria and therefore maintaining an optimal gut environment, resulting in beneficial effects for the host[1]. They are presently considered as an efficient strategy for the maintenance of gut health in humans and animals. The fermentation of fibre-rich substrates in the intestines selectively stimulates the proliferation and metabolic activity of health-associated beneficial bacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp.[2] and prevents the colonisation by potential pathogens[3] at the end of the small intestine and in the hindgut. The saccharolytic fermentation of dietary fibres by the endogenous microbiota yields SCFA among which butyrate helps creating and maintaining gut health and ecology. Butyrate is of special interest as it is the main source of energy for colonocytes[4,5], as it can decrease intestinal inflammation and as it enhances the intestinal barrier function[6]. Butyrate production mainly arises from the *Clostridium* clusters IV and XIVa via the butyryl-CoA:acetate-CoA transferase enzyme[7] which are therefore considered as health-associated bacteria[8].

The weaning period is a critical transitory phase in humans and animals[9]. It is characterised by a multifactorial stress, contributing to altered intestinal activities and health issues[10–12]. In pig production, the piglet's intestines become

Abbreviations: ADF, acid-detergent fibre; FS, fermentation supernatant; IPEC-J2, intestinal porcine epithelial cells; NDF, neutral-detergent fibre; qPCR, quantitative PCR.

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more susceptible to the invasion of pathogens, resulting in post-weaning diarrhoea. In this context, more attention has been paid to the presence of dietary fibres in the diet of young piglets as a strategy to reduce post-weaning-associated disorders and thus the need for antibiotics. Inulin is currently acknowledged as an efficient prebiotic for young mammals[13,14]. Some agricultural and industrial by-products such as chicory root and pulp contain significant amounts of inulin even after undergoing the extraction process[15]. Similarly, remaining pectin can be found in citrus peels[16], while rye bran is rich in arabinoxylans and soya-oligosaccharides are present in soya hulls[17]. The potential prebiotic function of such fibre-rich by-products, considered as more economic and sustainable than inulin, is therefore of great interest.

Until now, several *in vitro* gut models investigated the properties of prebiotics[18–20] or probiotics[21,22] on the intestinal epithelial cells. In addition to their well-documented prebiotic activities, these fibre-rich ingredients could exert an indirect effect, through microbiota shifts and/or the production of fermentation metabolites[23], which at their turn may result in an effect on intestinal inflammation and gut integrity. Owing to the complexity of the intestinal environment, arising from complex interactions between the host, the microbiota and the fermentation end products, it is valuable to consider a holistic approach to study the immunomodulatory and anti-inflammatory properties of prebiotics that were submitted to a colonic fermentation[23]. Therefore, we should consider an approach combining an *in vitro* gut fermentation model with pig faeces[24,25] with an *in vitro* intestinal cell culture experiment. Intestinal porcine epithelial cells (IPEC-J2) are one of the few cell lines that is not derived from tumour origin and can be polarised into monolayer epithelial cells[26,27]. They have been used for prebiotic and probiotic investigations and may therefore provide insights into metabolites and microbiota interactions with the intestinal mucosa. Although the IPEC-J2 cell line is derived from the small intestine of young piglets, no colonic cell line from porcine origin is available. In this way, the *in vitro* technique used in the present study simulated the interplay between the ingredients, the microbiota and the metabolites representing the intestinal chyme and cultured IPEC-J2 mimicking the intestinal lumen in the small intestine. The interest of the complete fermentation model taking into account both bacteria and metabolites could arise from the production of potential cytoprotective bacterial metabolites such as SCFA, and especially butyrate, which have the ability to modulate gene expression[28,29] as well as the protective effect of the microbiota itself interacting directly with the intestinal epithelial cells.

In the present study, chicory root and pulp, citrus pulp, rye bran and soya hulls that had been previously selected amongst various sources of carbohydrate-rich by-products based on an *in vitro* fermentation model were further tested for their prebiotic activities. The objective of the present study was to assess if the different sources of feed by-products reached the same prebiotic potential as inulin, considered as a positive control, in terms of fermentation characteristics and to compare the immunomodulatory profiles of the fermentation supernatants (FS) on cultured IPEC-J2.

Methods

Dietary fibre source

Six feed ingredients were chosen to represent a wide range of dietary fibre sources with a potential for inclusion in the weaning piglet's diet (Table 1).

Table 1. Chemical composition (g/kg DM) of the ingredients and total constituent monosaccharide composition of the non-cellulosic polysaccharide fraction

Chemical composition (g/kg DM)	Inulin	Chicory root	Chicory pulp	Rye bran	Soya hulls	Citrus pulp
DM (g/kg)	953	923	870	905·3	912·3	901
Fat	–	6·7	17·3	22·9	10·1	29·3
Protein	–	62·2	75·1	150·9	100·4	73·1
Ash	0·7	50·0	70·0	32·6	44·2	66·3
NDF*	4·3	62·0	320·0	184·6	478·8	240·9
ADF†	1·2	55·0	240·0	82·9	499·1	240·7
Fructan	894·2	664·3	156·1	50·8	2·8	17·1
Total AX				199·1		
WU-AX				166·8		
WE-AX				32·3		
A/X (no unit)				0·5		
AGA						96·6
AGU						24·3
Carbohydrate composition						
Rhamnose	4·8	2·7	9·3	–	6·9	7·7
Arabinose	2·3	16·3	80·9	79·6	49·0	78·6
Xylose	1·8	4·7	19·1	142·7	62·7	36·5
Mannose	95·4	77·2	18·1	12·7	36·7	11·1
Glucose	162·2	127·7	27·4	391·05	20·9	119·2
Galactose	7·7	14·9	45·4	23·27	27·0	47·0
Fucose					0·1	0·1
Raffinose					1·1	–
Stachyose					4·9	0·5
Verbascose					1·2	1·0

NDF, neutral-detergent fibre; ADF, acid-detergent fibre; AX, arabinoxylan; WU-AX, water-unextractable arabinoxylan; WE-AX, water-extractable arabinoxylan, AGA, galacturonic acid; AGU, glucuronic acid.
* Hemicellulose + cellulose + lignin.
† Cellulose + lignin.

Inulin (Fibruline Instant®), chicory root (Fibrofos 60®) and chicory pulp were provided by Cosucra Groupe Warcoing SA (Warcoing, Belgium). Citrus pulp (mix of albedo and flavedo), rye bran and soya hulls were obtained from a commercial supplier (Royal Agrifirm Group, Apeldoorn, The Netherlands). The ingredients were selected according to a previous screening of several sources of by-products differing in structural carbohydrates, based on their fermentation characteristics, that is, SCFA and microbiota profiles. An overview of the bacterial populations present in the FS of all tested ingredients is presented in online Supplementary Fig. S1 and was mainly used as selective criteria for the present study, along with the previous data[30].

Analysis of dietary fibre sources

The ingredients were analysed for organic matter (AOAC 923.03), DM (AOAC 967.03), crude protein (Foss Kjeltec Analyzer Unit 2300; CP = N × 6·25), fat content (Soxhlet method; AOAC 920.29) and neutral-detergent fibre (NDF) and acid-detergent fibre (ADF) (Foss Fibrecap system; Van Soest *et al.*[31]). Non-cellulosic total monosaccharide composition was determined according to the method of Englyst & Cummings (1984)[32] adapted by Aguedo *et al.* (2014)[33]. The uronic acids were detected by high-performance anion-exchange chromatography with pulsed amperometric detection[33]. The fructan amount was assessed by size-exclusion HPLC as previously described[33]. Total arabinoxylan content and water-extractable arabinoxylan content were measured according to the method of Gebruers *et al.*[34] using GC (Agilent Technologies).

In vitro *digestion and batch fermentation of dietary fibre sources*

The ingredients were studied using a modified three-step *in vitro* model of the pig's gastro-intestinal tract[35] combining an enzymatic hydrolysis and dialysis to a batch fermentation with faecal microbiota. The grinded ingredients underwent a pepsin-pancreatin hydrolysis adapted from the protocol of Boisen & Fernández[36] as described by Uerlings *et al.*[30] followed by a dialysis step according to Kalala *et al.*[37].

For the batch fermentation, a faecal inoculum was prepared from a buffer solution composed of salts and minerals devoid of reducing agent (pH 6·8; Menke & Steingass[38]) and mixed frozen faeces (2·5 %, w/v) from piglets under anaerobic conditions (Invivo$_2$, Led Techno) with three mucin microcosms (K1-carrier, AnoxKaldnes AB) per fermentation vial[39], with the hydrolysed ingredient or not (blank vials).

Williams *et al.*[24] reported that faeces are a suitable and representative inoculum to mimic the *in vivo* gut fermentation which justifies the model chosen for the following research. Faeces were previously collected from pre-weaned 3 week-old-piglets (male and female) by faecal stimulation with sterile swabs. The sows and the piglets were housed in individual farrowing units, equipped with wood shavings as litter, and the piglets had a space with a heat lamp. There was no creep feed available to the piglets, and they were thus only consuming milk from their mother. All experimental procedures led on piglets (faeces collection) were in accordance with European and Belgian regulations concerning the care and use of animals for research purposes and were approved by the Animal Ethical Committee of Liège University, Belgium (protocol number: 1860).

Each ingredient was added in three vials for gas measurements, six vials for SCFA measurements at each time point, three vials for microbiota measurements at each time point and three vials for the cell culture assay. The different vials were placed into an agitating water bath at 39°C with 50 rpm agitation, and the fermentation broth was stored at −80°C.

Sampling times for SCFA and microbiota population measurements were based on the hindgut transit time in the large intestine of growing pigs[40]. As substrate depletion is one of the limitations of the *in vitro* batch fermentation model, SCFA production and microbiota composition were assessed after 6, 12 and 24 h, with a limited decline in bacterial population (data not shown).

Fermentation kinetics profile of the in vitro *batch fermentation*

The released gas volumes were repeatedly recorded with a Tracker 200 manometer (Bailey & Mackey Ltd) with a needle of 0·6 × 25 mm at following time points: 2, 5, 8, 12, 16, 20, 24, 48 and 72 h according to the model of Groot *et al.*[41] (*n* 3 fermentation vials), and gas production recordings were fitted to the mathematical monophasic model:

$$G = \frac{A \times t^C}{t^C + B^C}$$

$$R_{MAX} = \frac{A \times B^C \times C \times T_{MAX}{}^{-c-1}}{(1 + B^C \times T_{MAX}{}^{-C})^2}$$

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

with A (ml/g DM) as the maximum gas volume, G (ml/g DM) as the gas accumulation to time, B (h) as the time to half asymptote when G = A/2, R_{MAX} as the maximum rate of gas production (ml/g DM × h) and T_{MAX} as the time to reach R_{MAX}.

Fermentation products profile of supernatants from in vitro *batch fermentation*

Fermentation broths sampled after 6, 12 and 24 h of fermentation (*n* 6 fermentation vials) were analysed by isocratic HPLC using the Alliance System e2695 (Waters) with an Aminex HPx-87H column (BioRad) as described by Uerlings *et al.*[30]. A calibration curve with known concentrations of SCFA and lactate was used to quantify the amounts present in the samples.

Microbiota composition of supernatants from in vitro *batch fermentation*

Microbiota profile kinetics were measured after 6, 12 and 24 h of fermentation (*n* 3 fermentation vials). Genomic DNA from fermentation broth samples was extracted using the QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's instructions adapted by Uerlings *et al.*[30]. The DNA

Table 2. Nucleotide sequences of primers for the microbiota composition of fermentation supernatant

Target gene		Primer sequence 5′→3′	Reference	Primer concentration (nM)
Total bacteria	Unibac-F	CGTGCCAGCCGCGGTAATACG	(42)	600
	Unibac-R	GGGTTGCGCTCGTTGCGGGACTTAACCCAACAT		
Lactobacillus spp.	LAA-F	CATCCAGTGCAAACCTAAGAG	(44)	300
	LAA-R	GATCCGCTTGCCTTCGCA		
Bifidobacterium spp.	Bif164-F	GGGTGGTAATGCCGGATG	(45)	300
	Bif662-R	CCACCGTTACACCGGGAA		
Clostridium cluster IV	sg-Clept-F	GCACAAGCAGTGGAGT	(46)	300
	sg-Clept-R3	CTTCCTCCGTTTTGTCAA		
Clostridium cluster XIVa	g-Ccoc-F	AAATGACGGTACCTGACTAA	(47)	300
	g-Ccoc-R	CTTTGAGTTTCATTCTTGCGAA		
Butyryl-CoA:acetate-CoA transferase	F	TGGACAGAAAGGTTGCGGAG	(30)	300
	R	GTGTGTACGCCCAGATCCTT		

concentration and quality were, respectively, determined by Nanodrop (Thermofisher Scientific Nanodrop 2000) and agarose gel (1 %).

Quantitative PCR (qPCR) was performed on DNA samples to quantify *Lactobacillus* spp., *Bifidobacterium* spp., *Clostridium* clusters IV and XIVa, total bacteria as well as the butyryl-CoA: acetate-CoA transferase gene abundance. Real-time PCR analysis was conducted using the StepOne Plus (ThermoFisher Scientific) using SYBR Premix Ex Taq, Tli RNase H Plus (Takara Bio Inc. Ltd). The commercially manufactured gene specific primers are shown in Table 2 (Eurogentec). qPCR conditions were optimised to obtain primer efficiency values between 90 and 110 %. All runs were performed with the default protocol, with a pre-denaturation step (30 s, 95°C) followed by amplification for forty cycles with a denaturation step (5 s, 95°C), an annealing step (1 min, 60°C) and an extension step (30 s, 72°C). Primers specificity was verified through melting curves. Total bacteria[42] was selected as a reference gene after verification of the stability for all experimental conditions. For each target gene, the relative gene abundance level was calculated by the $2^{-\Delta\Delta ct}$ method[43] using a pooled sample as an internal control.

Fermentation supernatant preparation

The FS of the five ingredients and inulin after 12 h of fermentation (pooled FS coming from three different fermentation vials) were sterile-filtered with 0·22-µm ø pore diameter (Filter Service) for the cell proliferation assay ('sterile-filtered FS') and with 0·8-µm pore ø ('complete FS'), to remove the matrix debris for the cell response assay by high-throughput qPCR.

Intestinal porcine epithelial cell line and culture conditions

The non-transformed porcine intestinal epithelial cell line (IPEC-J2), originally isolated from jejunal epithelia of a neonatal unsuckled piglet[48], was grown at 37°C in a humidified atmosphere of 5 % CO_2 in complete Dulbecco's modified Eagle's medium (DMEM)/F-12, supplemented with 1 % penicillin–streptomycin, 5 % fetal bovine serum, 2 mM L-glutamine, 5 ng/ml epidermal growth factor, 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml Se (all from Sigma). Plain medium was added once every 2 d, and cells were passaged when they reached confluence.

Modulation of intestinal porcine epithelial cell viability by fermentation supernatant

Cell viability was used to determine the concentration of FS to be applied for the cell response assay of testing the impact of FS on gene expression in IPEC-J2. Cell proliferation was measured with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. IPEC-J2 cells between passages 15 and 20 were seeded in ninety-six-well flat bottomed plates at a density of 20 000 cells/100 µl (100 µl/well). Cells were allowed to adhere for 24 h until confluence was reached and were re-fed with experimental media without antibiotics before being treated with different concentrations of 0·22-µm ø sterile-filtered FS (50, 25, 10, 5, 2·5 %, v/v). After incubation with different concentrations of FS for 24 h, the culture medium was removed. Next, fresh antibiotic-free culture medium and 15 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (Promega) were added to each well for another 4 h at 37°C prior to measurement of cell viability. The absorbance at 570 nm was determined in a micro-plate reader (VICTOR plate reader, PerkinElmer). There were six well-replicates per treatment. According to the cell viability test, a concentration of 10 % (v/v) was chosen to study the impact of FS on gene expression in IPEC-J2 cells.

Impact of fermentation supernatant on gene expression in intestinal porcine epithelial cells

IPEC-J2 cells between passages 15 and 20 were seeded in 24-well plates at a density of $2{\cdot}5 \times 10^5$ cells/ml (1 ml/well). Prior to treatment, confluent monolayers of the IPEC-J2 cells were washed with plain medium without antibiotics. FS (0·8-µm ø filtered) was applied (10 %, v/v) for 24 h. For sham-stimulation, cells were maintained in the culture medium for 24 h. After washing with PBS, lysis buffer (RNeasy Mini Kit, Qiagen) supplemented with β-mercaptoethanol (Sigma) was added to the IPEC-J2 cells and lysates were collected and kept at −80°C until further processing. There were three well-replicates per treatment.

Total RNA from IPEC-J2 cells treated with 0·8-μm ø FS was extracted using an RNeasy Mini kit (RNeasy Mini Kit), according to the manufacturer's protocol. RNA concentration and quality were determined by Nanodrop (Thermofisher Scientific Nanodrop 2000, Thermo Fisher Scientific) and agarose gel (1 %), respectively. Extracted RNA was converted into cDNA by reverse transcription of 60 ng total RNA using a Reverse Transcription Master Mix (Fluidigm Corporation) and analysed by high-throughput qPCR as described by Stoy *et al.*[49].

Briefly, pre-amplification was performed according to the PreAmp MasterMix manufacturer's instructions (Fluidigm Corporation) followed by an exonuclease I treatment (New England Biolabs).

Intron spanning primer pairs, yielding a PCR product lower than 150 bp, were designed using Primer-BLAST (NCBI) and were validated through agarose gel electrophoresis and through melting curves (Table 3). Pooled pre-amplified cDNA samples with 3-fold dilution series were used to obtain primer efficiency.

Table 3. Primer sequences for the gene expression levels of intestinal porcine epithelial cells treated with fermentation supernatants

Target family	Target gene		Primer sequence 5′→3′
Housekeeping genes	*ACTB*	F	CTACGTCGCCCTGGACTTC
		R	GCAGCTCGTAGCTCTTCTCC
	B2M	F	ACCACTTTTCACACCGCTC
		R	GCTTTCCGTTTTCCGCTGG
	ESPN	F	CACTGGCAAAGTGAGAGTCCT
		R	TGTGGTCAGCCCCTTACTCT
	GAPDH	F	GATGGTGAAGGTCGGAGTGAA
		R	GTGGAGGTCAATGAAGGGGT
	HBMS	F	CCTTTAGCGGGGGAAATCAC
		R	CTGAAGCCCATCCCAGCTAA
	HPRT1	F	AATTCTTTGCTGACCTGCTGGA
		R	TCCACCAATTACTTTTATATCGCCC
	PCNA	F	CTGCAAGTGGAGAACTCGGAA
		R	AAGTTCAGGTACCTCAGTGCAA
	PPIA	F	GGGACCTGGAAACCAAGAAGTG
		R	ACTTTGTCTGCAAACAGCTCCAATC
	RPL13a	F	ATTGTGGCCAAGCAGGTACT
		R	AATTGCCAGAAATGTTGATGC
	RPL32	F	GCTTGAAGTGCTGCTAATGTG
		R	GGATTGGTGACCCTGATGGC
	RPL4	F	GAGAAACCGTCGCCGAATCC
		R	CCCACCAGGAGCAAGTTTCAA
	SDHA	F	GTCGTCGGCCAAAGTTTCAG
		R	TGTGTTAAACCCGGCCTCAG
	TBP	F	CGGACCACCGCACTGATATT
		R	TTCTTCACTCTTGGCTCCCG
	YWHAZ	F	TTGTAGGAGCCCGTAGGTCA
		R	AGCACCTTCCGTCTTTTGCT
Inflammation signalling pathway genes	*AKT1*	F	CTAAGCCCAAACACCGCGT
		R	TCAGGATCTTCATGGCGTAGT
	MAPK14	F	TACCCGAGCGTTACCAGAAC
		R	TTCACTGCAACACGTAACCCA
	MyD88	F	GCATCACCATTCGAGATGACC
		R	TCCTGCACAAACTGGGTATCG
	NF-kB1	F	AAGAAGTCCTACCCTCAGGTCA
		R	CAGTGACAGTGCAGATCCCA
	NF-kBIα	F	GAGGATGAGCTGCCCTATGAC
		R	CCATGGTCTTTTAGACACTTTCC
	NOD1	F	GTCGTCAACACCGATCCAGT
		R	CCTCCTTCTGGGCATAGCAC
	PPARγ	F	ACAGCGACCTGGCGATATTTA
		R	GAGGACTCTGGGTGGTTCAA
Pro-inflammatory genes	*CCL5*	F	ACACCACACCCTGCTGTTTT
		R	TCTTCTCTGGGTTGGCACAC
	COX2	F	TCGAGATGATCTACCCGCCT
		R	ACATCATCAGACCAGGCACC
	CXCL10	F	CCCACATGTTGAGATCATTGC
		R	GCTTCTCTCTGTGTTCGAGGA
	DEFβ1	F	TTCCTCCTCATGGTCCTGTTAC
		R	CCACAGGTGCCGATCTGTTTC
	DEFβ4a	F	CAGGATTGAAGGGACCTGTT
		R	CTTCACTTGGCCTGTGTGTC
	IFNβ	F	TTCGAGGTCCCTGAGGAGATT
		R	GCTGGAGCATCTCGTGGATAA
	IL1β	F	CCAAAGAGGGACATGGAGAA
		R	GGGCTTTTGTTCTGCTTGAG
	IL18	F	CTGAAAACGATGAAGACCTGGA
		R	CCTCAAACACGGCTTGATGTC
	IL6	F	TGGGTTCAATCAGGAGACCT
		R	CAGCCTCGACATTTCCCTTA
	IL8	F	GACTTCCAAACTGGCTGTTGC
		R	ATTTGGGGTGGAAAGGTGTG
	ILRN1	F	TGCCTGTCCTGTGTCAAGTC
		R	GTCCTGCTCGCTGTTCTTTC
	MCP1	F	CTCACTGCAGCCACCTTCT
		R	CACTTGCTGCTGGTGACTCT
	TNFα	F	TCTGCCTACTGCACTTCGAG
		R	GTTGATGCTCAAGGGGCCA
Intestinal barrier integrity genes	*CASP3*	F	AAGCAAATCAATGGACTCTGGAA
		R	TTGCAGCATCCACATCTGTACC
	CDH1	F	AGCCCTGCAATCCTGGCTTT
		R	AGAAACATAGACCGTCCTTGGC
	Claudin-1	F	GGTGACAACATTGTGACGGC
		R	TACCATCAAGGCACGGGTTG
	Claudin-3	F	TATCACAGCGCGGATCACC
		R	CTCTGCACCACGCAGTTCAT
	Claudin-4	F	CTTCATCGGCAGCAACATCG
		R	CGAGTCGTACACCTTGCACT
	EGFR	F	GCACAAGGACAACATCGGCTC
		R	GATCTTGACATGCTGCGGTGT
	MARVELD2	F	CTCAGCCCCGCCATTACCTG
		R	TAGAGGTGATGTGCTGTTGCC
	MUC1	F	GGATTTCTGAATTGTTTTTGCAG
		R	ACTGTCTTGGAAGGCCAGAA
	Occludin	F	AACGTATTTATGACGAGCAGCCC
		R	CACTTTCCCGTTGGACGAGTA
	TGFβ1	F	CATTCACGGCATGAACCGGC
		R	CGCACGCAGCAGTTCTTCTC
	VIL1	F	ACAAAGGTCGCTGTCCTCCA
		R	TGACCTGGGCGTTCAGTTTG
	ZO-1	F	AAGGTCTGCCGAGACAACAG
		R	TCACAGTGTGGTAAGCGCAG

ACTB, actin beta; *B2M*, beta-2-microglobulin; *ESPN*, espin; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HBMS;* hydroxymethylbilane synthase; *HPRT1*, hypoxanthine phosphoribosyltransferase 1; *PCNA*, proliferating cell nuclear antigen; *PPIA*, peptidylprolyl isomerase A; *RPL*, ribosomal protein L; *SDHA*, succinate dehydrogenase complex, subunit A; *TBP*, TATA box binding protein; *YWHAZ*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta; *AKT1*, serine/threonine-protein kinase 1; *MAPK14*, mitogen-activated protein kinase 14; *MyD88*, myeloid differentiation primary response 88; *NF-kBIα*, NF-κB inhibitor alpha; *NOD1*, nucleotide-binding oligomerisation domain-containing protein 1; *TLR*, toll-like receptor; *CCL5*, chemokine ligand 5; *COX2*, cyclo-oxygenase 2; *CXCL10*, C-X-C motif chemokine 10; *DEFβ*, defensin beta; *EGFR*, epidermal growth factor receptor; *IFN*, interferon; *ILRN1*, IL-1 receptor antagonist; *MCP1*, monocyte chemoattractant protein 1; *CASP3*, caspase 3; *CDH1*, E-cadherin; *MARVELD2*, tricellulin; *MUC1*, mucin 1; *TGFβ1*, transforming growth factor beta 1; *VIL1*, villin 1; *ZO-1*, zonula occludens-1.

Results are shown for those that had an appropriate primer efficiency, between 90 and 110 %. High-throughput qPCR was performed in 96 × 96 dynamic array integrated fluidic circuits (Fluidigm Corporation). After loading, the dynamic array was placed in a BioMark HD Real-Time PCR System (Fluidigm Corporation) and the following cycle parameters were used: 60 s at 95°C, followed by thirty cycles with denaturing for 5 s at 96°C, and annealing/elongation for 20 s at 60°C. Reactions were performed in six replicates (cDNA replicates). Non-template controls were included to reflect nonspecific amplification or sample contaminations.

Quantification cycles (*Cq*) were acquired using the Fluidigm real-time PCR analysis software 3.0.2 (Fluidigm Corporation). The geometric mean of four reference genes (ribosomal protein L 13a (*RPL13a*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), peptidylprolyl isomerase A (*PPIA*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*)) was used to normalise samples. These genes were found to be stably expressed reference genes across 0·8-µm ø filtered supernatants (of inulin, chicory root, chicory pulp, rye bran, soya hulls and citrus pulp) using NormFinder[50]. For each target, the relative gene expression levels were calculated by the $2^{-\Delta\Delta ct}$ method[43]. However, gene expression was different between the control treatment sham-treated cells and the 0·8-µm ø FS treatments for the eleven reference genes studied. Therefore, the different fermented ingredients were compared with inulin (considered as a positive control in this case).

Statistical analysis

Homogeneity between variances and normality among treatments was confirmed using, respectively, Bartlett's and Ryan-Joiner's tests. The experimental units for the fermentation parameters and for the immunomodulatory parameters were the fermentation vial and the cell culture wells, respectively. The experimental data concerning gas production and high-throughput qPCR data were subjected to GLM procedures, and the significantly different means were identified by *post-hoc* Tukey's multiple range HSD test using SAS 9.4 software (SAS Institute). The procedure included one fixed criteria of classification (type of ingredient). For the high-throughput qPCR data, adjusted *P*-values were obtained using a false discovery rate correction with the linear method of Benjamini and Hochberg. The analyses of SCFA and microbial communities were performed similarly. However, the procedure included two fixed criteria of classification (type of ingredient and sampling time) as well as their interaction. For SCFA and microbiota profiles, when a significant interaction of a time effect was encountered, parameters were studied by one-way ANOVA per time point. Previous *in vitro* trials were used to validate the sample size of the present study based on similar variables such as microbiota and SCFA analyses arising from *in vitro* batch fermentations[39,51,52] as well as IPEC-J2 investigations[53,54]. *P*-values <0·05, <0·01 and <0·001 were considered as statistically significant, highly significant and very highly significant, respectively.

Results

Fermentation kinetics profile of the in vitro batch fermentation

Inulin and chicory root contained high amounts of fructans (89·4 and 66·4 %, respectively), whereas chicory pulp was characterised by a low fructan amount and high NDF and ADF levels (Table 1). Glucose was the most abundant building block of the non-cellulosic fraction followed by mannose for inulin and chicory root and by arabinose for chicory pulp (Table 1). With a lower fructan amounts (5·1 %), rye bran was characterised by elevated levels of arabinoxylans, of which the main water-unextractable arabinoxylans fraction is majoritarian and consequently displayed high levels of glucose, arabinose and xylose monosaccharides after hydrolysis. Soya hulls had the highest NDF and glucose monosaccharide levels. Citrus pulp displayed intermediate NDF levels, a high pectin content and was mainly composed of glucose and arabinose (Table 1).

According to the cumulative gas production, chicory root along with inulin, considered as a positive control, induced an extensive fermentation (greatest total gas production; A) in comparison with the other ingredients ($P < 0·0001$; Table 4). With the lowest half-time to asymptotic gas production (B), the highest rate of fermentation (R_{MAX}) and the lower time to reach R_{MAX} (T_{MAX}), chicory root was the most rapidly fermented feed ingredient after inulin (Table 4). Chicory pulp and citrus pulp demonstrated intermediate fermentation

Table 4. Gas fermentation parameters (A, B, R_{MAX}, T_{MAX}) modelled according to Groot *et al.*[41] of feed ingredients in the presence of faecal inoculum of pre-weaned 3-week-old-piglets (*n* 3 fermentation vials)*
(Mean values with their standard errors)

	A (ml/g DM)		B (h)		R_{MAX} ml/(g DM × h)		T_{MAX} (h)	
Ingredients	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Inulin	316[a]	7	8·1[d]	0·1	26·9[a]	0·9	5·2[c]	0·1
Chicory root	291[a,b]	10	8·2[d]	0·1	22·9[b]	0·5	4·7[c]	0·1
Chicory pulp	256[b,c]	7	12·1[b]	0·3	14·2[c]	0·3	7·6[a]	0·4
Rye bran	214[d]	8	10·7[c]	0·1	12·6[c]	0·5	5·3[b,c]	0·3
Soya hulls	262[b,c]	7	17·1[a]	0·6	9·4[d]	0·5	7·1[a,b]	0·7
Citrus pulp	243[c,d]	10	12·8[b]	0·2	12·8[c]	0·6	8·1[a]	0·3
P	<0·0001		<0·0001		<0·0001		<0·0001	

[a,b,c,d] Mean values within a column with unlike superscript letters are significantly different ($P < 0·05$).
* A, total gas produced; B (h), time to half asymptote; R_{MAX}, maximum rate of gas; T_{MAX}, time at which R_{MAX} is reached. Gas production values were recorded over 72 h using a manometer.

kinetics. Rye bran, fermented in a slow (intermediate B and T_{MAX}) and less extensive (low R_{MAX}) manner, reached the lowest maximal gas production (A) ($P < 0{\cdot}0001$; Table 4). Although an intermediate total gas production (A) was recorded for soya hulls (similar to the ones of chicory pulp, root and citrus pulp), this ingredient demonstrated the slowest rate of fermentation (R_{MAX}; Table 4).

Fermentation products profile of supernatants from in vitro *batch fermentation*

The interaction between ingredients and the time of fermentation were significant for all the measured metabolites (Table 5). Acetate and butyrate molar ratios at 6 h were equal to zero for all ingredients, explaining the extremely high percentages of BCFA encountered (Table 5). Total SCFA amounts as well as propionate and BCFA ratios were similar at 6 h. Lactate levels encountered for the major part of the produced metabolites with inulin displaying the greatest amounts followed by chicory root ($P < 0{\cdot}0001$). Inulin and soya hulls displayed higher amounts of total SCFA at 12 h. Fermentation of ingredients resulted in different ($P < 0{\cdot}001$; Table 5) butyrate net production at this time point with inulin, chicory root and rye bran displaying the highest net molar ratio of butyrate (% of total SCFA), although the total SCFA production of rye bran was the smallest. Reflecting the SCFA profile at 12 h, inulin, chicory by-products and soya hulls displayed the highest SCFA amounts at 24 h of fermentation, while rye bran was the smallest producer of SCFA. Inulin displayed the highest butyrate ratio, followed by chicory root. Alternatively, chicory pulp and citrus pulp were demonstrating higher acetate molar ratios compared with inulin and were correspondingly among the lowest butyrate producers ($P < 0{\cdot}0001$; Table 5).

Microbiota composition of supernatants from in vitro *batch fermentation*

Broth from fermented citrus pulp displayed the highest levels of *Clostridium* cluster IV at 6 h compared with inulin, while the other ingredients demonstrated intermediate values ($P < 0{\cdot}001$; Fig. 1(A)). At 24 h, citrus pulp as well as chicory pulp and soya hulls induced the greatest abundance in *Clostridium* cluster IV. Citrus pulp exhibited the highest levels of *Clostridium* cluster XIVa, whereas inulin displayed the lowest levels at 24 h ($P < 0{\cdot}001$; Fig. 1(B)). Butyryl-CoA:acetate-CoA transferase gene abundance was significantly higher in soya hulls compared with the other ingredients at 6, 12 and 24 h except for rye bran which reached the same levels as soya hulls after 12 h ($P < 0{\cdot}001$; Fig. 1(C)). *Lactobacillus* spp. gene abundance greatly differed with the time of fermentation (Fig. 1(D)). Although several FS such as rye bran, chicory pulp, soya hulls and citrus pulp demonstrated the highest abundance of *Lactobacillus* spp. at 6 and 12 h, in contrast with inulin and chicory root, no difference was perceived between ingredients after 24 h of fermentation ($P < 0{\cdot}001$; Fig. 1(D)). The greatest abundance of *Bifidobacterium* spp. was observed in soya hulls at 6 h and in rye bran fermented broths at 12 and 24 h of fermentation (Fig. 1(E)).

Table 5. Fermentation product profile of the fermentation supernatant of the different ingredients after 6, 12 and 24 h of fermentation (*n* 6 fermentation vials)*
(Mean values of six measurements with their standard errors)

		Lactate (mmol/g OM)		Total SCFA (mmol/g OM)		Acetate (%)		Propionate (%)		Butyrate (%)		BCFA (%)	
Time (h)	Ingredients	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
6	Inulin	$2{\cdot}23^{a}$	0·12	0·97	0·21	0·00	0·00	37·58	8·21	0·00	0·00	62·42	8·21
	Chicory root	$1{\cdot}74^{b}$	0·16	0·73	0·21	0·00	0·00	38·66	12·48	0·00	0·00	61·30	12·50
	Chicory pulp	$0{\cdot}49^{c,d}$	0·01	0·99	0·09	0·00	0·00	24·65	5·86	0·00	0·00	75·02	6·10
	Rye bran	$0{\cdot}69^{c}$	0·04	0·99	0·22	0·00	0·00	26·04	11·35	0·00	0·00	73·96	11·35
	Soya hulls	$0{\cdot}28^{d}$	0·01	1·13	0·20	0·00	0·00	24·01	4·70	0·00	0·00	75·99	4·70
	Citrus pulp	$0{\cdot}46^{c,d}$	0·07	1·12	0·30	0·00	0·00	14·38	5·03	0·00	0·00	85·62	5·03
12	Inulin	$1{\cdot}79^{a}$	0·44	$4{\cdot}27^{a}$	0·43	$41{\cdot}06^{a,b,c}$	1·73	$51{\cdot}87^{a,b}$	1·52	$6{\cdot}29^{a}$	0·70	0·76	0·47
	Chicory root	$0{\cdot}44^{b}$	0·15	$1{\cdot}84^{b}$	0·15	$49{\cdot}20^{a,b}$	1·97	$45{\cdot}54^{b,c}$	2·20	$4{\cdot}97^{a,b}$	0·44	0·27	0·12
	Chicory pulp	$0{\cdot}01^{b}$	0·01	$2{\cdot}00^{b}$	0·14	$55{\cdot}82^{a}$	1·16	$41{\cdot}03^{b,c}$	1·30	$2{\cdot}33^{b,c}$	0·64	0·80	0·72
	Rye bran	$0{\cdot}08^{b}$	0·06	$1{\cdot}10^{b}$	0·19	$38{\cdot}59^{b,c}$	2·49	$48{\cdot}30^{a,b,c}$	1·90	$3{\cdot}31^{a,b,c}$	0·55	9·78	1·14
	Soya hulls	$0{\cdot}00^{b}$	0·00	$3{\cdot}97^{a}$	0·44	$28{\cdot}09^{c}$	7·18	$61{\cdot}48^{a}$	6·72	$2{\cdot}24^{b,c}$	1·17	8·17	1·01
	Citrus pulp	$0{\cdot}05^{b}$	0·02	$1{\cdot}80^{b}$	0·26	$48{\cdot}00^{a,b}$	4·98	$35{\cdot}72^{c}$	2·34	$1{\cdot}61^{c}$	1·21	14·65	0·76
24	Inulin	0·00	0·00	$3{\cdot}51^{a,b,c}$	0·96	$35{\cdot}51^{c}$	3·80	39·49	3·80	$18{\cdot}42^{a}$	2·04	$6{\cdot}56^{a}$	0·36
	Chicory root	0·00	0·00	$5{\cdot}24^{a}$	0·18	$38{\cdot}47^{b,c}$	9·38	47·78	9·38	$12{\cdot}01^{b}$	1·87	$1{\cdot}73^{b}$	0·68
	Chicory pulp	0·00	0·00	$4{\cdot}27^{a,b}$	0·62	$64{\cdot}24^{a}$	0·44	28·42	0·44	$5{\cdot}60^{c}$	0·11	$1{\cdot}73^{b}$	0·26
	Rye bran	0·02	0·01	$1{\cdot}42^{c}$	0·15	$50{\cdot}20^{a,b,c}$	2·31	38·94	2·31	$7{\cdot}46^{b,c}$	0·49	$3{\cdot}38^{b}$	0·44
	Soya hulls	0·02	0·01	$3{\cdot}80^{a,b}$	0·50	$52{\cdot}76^{a,b,c}$	5·52	40·39	5·52	$4{\cdot}31^{c}$	0·74	$2{\cdot}52^{b}$	0·57
	Citrus pulp	0·00	0·00	$2{\cdot}64^{b,c}$	0·26	$61{\cdot}78^{a,b}$	2·56	30·74	2·52	$5{\cdot}66^{c}$	0·49	$1{\cdot}81^{b}$	0·43
P-value ingredient		<0·0001		<0·0001		<0·0001		0·0026		<0·0001		<0·0001	
P-value time		<0·0001		<0·0001		<0·0001		<0·0001		<0·0001		0·0472	
P-value ingredient × time		<0·0001		<0·0001		<0·0001		0·0142		<0·0001		<0·0001	

SCFA, total amount of SCFA (acetic + propionic + *i*-butyric + butyric + *i*-valeric + valeric; expressed as mmol/g organic matter); acetic, propionic and butyric acid proportions (expressed as % of SCFA); OM, organic matter; BCFA, branched chain fatty acid proportion (*i*-butyric + *i*-valeric + valeric scaled to SCFA, expressed as %).
[a,b,c,d] For one sampling time, mean values within a column with unlike superscript letters are significantly different ($P < 0{\cdot}05$).
* Values after different fermentation times with different feed ingredients were corrected for fermentation products formed in the vials without ingredient and with mucin carriers added and are thus solely the result of fibre degradation (net values).

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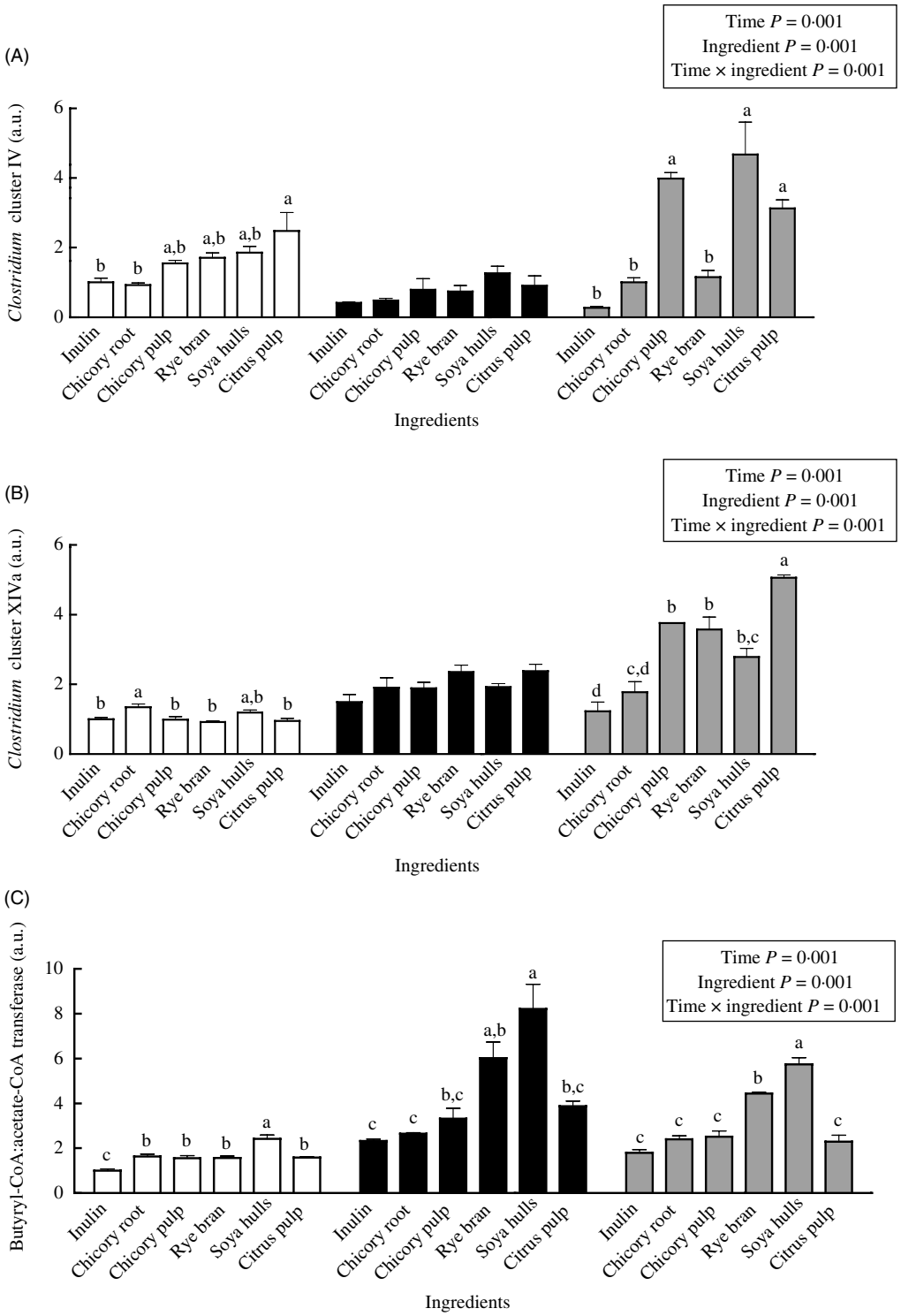

Fig. 1. Microbiota composition of supernatants after 6, 12 and 24 h of fermentation. (A) *Clostridium* cluster IV; (B) *Clostridium* cluster XIVa; (C) butyryl-CoA:acetate-CoA transferase; (D) *Lactobacillus* spp.; (E) *Bifidobacterium* spp. Values are means (n 3 fermentation vials) with their standard errors per bacterial group. [a,b,c,d]For one sampling time, mean values with unlike letters are significantly different ($P < 0{\cdot}05$). Total bacteria was selected as the reference and was stable across treatment. Inulin at 6 h was considered as control and was set at a value of 1·000. a.u., Arbitrary unit. □, 6 h; ■, 12 h; ▩, 24 h.

Modulation of intestinal porcine epithelial cell viability by fermentation supernatant

In order to choose the most appropriate concentration of FS for the IPEC-J2 model, a cell viability assay was conducted (Fig. 2). Sterile-filtered FS collected after 12 h of fermentation was not toxic for IPEC-J2 at a concentration <25 % (v/v) for most ingredients, with a reduction of the cell viability approximately 50 % at the cited concentration (EC_{50}). A concentration of 10 %

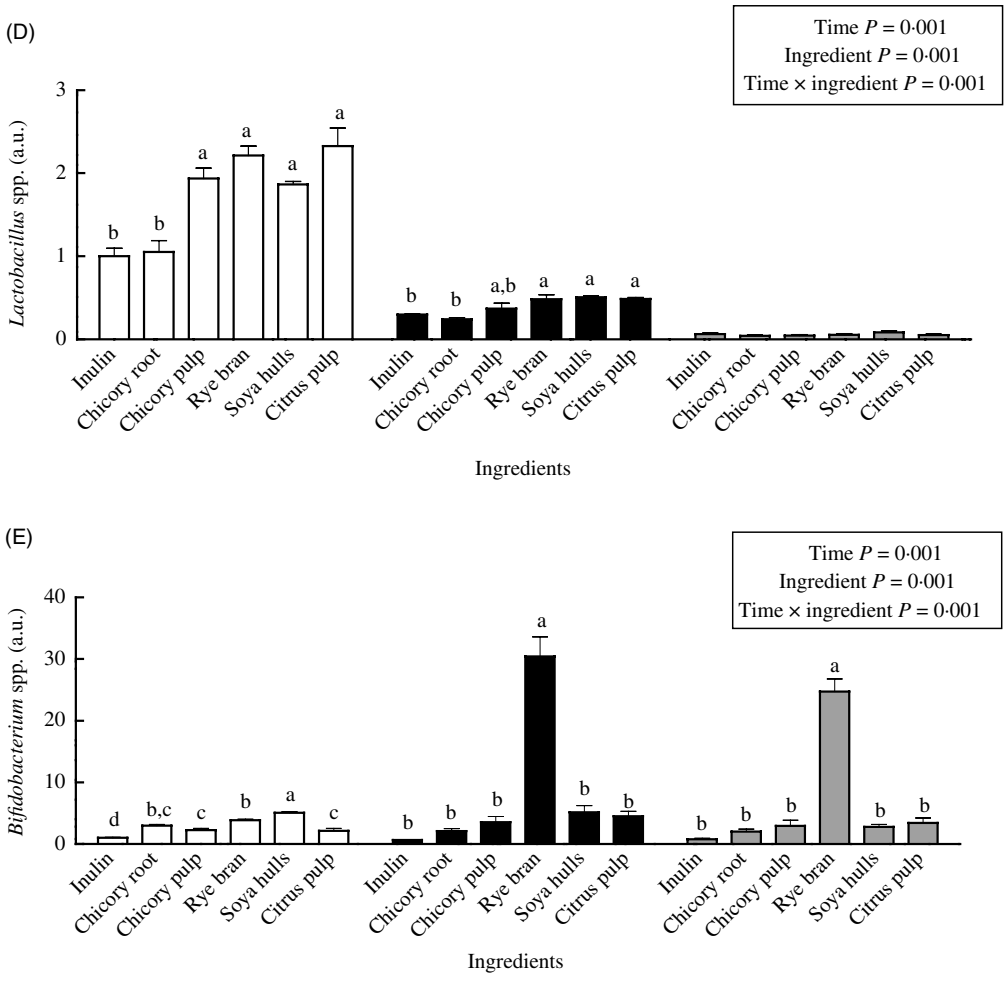

Fig. 1. (Continued).

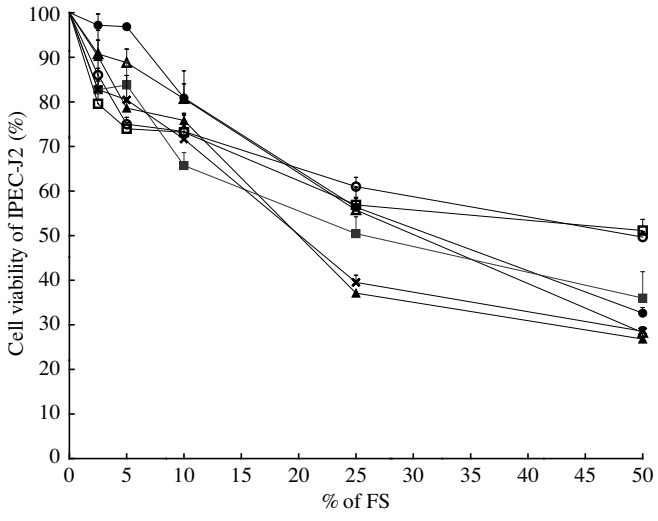

Fig. 2. Modulation of intestinal porcine epithelial cell (IPEC-J2) viability by fermentation supernatant (FS) collected after 12 h. Values are means of six well-measurements with their standard errors. -○-, Inulin; -●-, chicory root; -△-, chicory pulp; -▲-, rye bran; -□-, soya hulls; -■-, citrus pulp; -✕-, fermentation blank.

(v/v) led to a reduction of approximately 30 % of cell viability for all ingredients and the fermentation blank (Fig. 2). According to these results and the literature, 10 % (v/v) was chosen as a concentration for the immunomodulatory model.

Impact of fermentation supernatant on gene expression in intestinal porcine epithelial cells

High-throughput qPCR was performed with 0·8-µm ø filtered FS containing both metabolites and bacteria ('complete supernatant') which is representative of the *in vitro* gastro-intestinal model used in this research. Data revealed that none of the eleven reference genes studied was stable between the sham-treated cells and the cells receiving the FS (data not shown). Therefore, the fermented feed ingredients were compared with the acknowledged prebiotic control, that is, inulin to assess the immunomodulatory effect of their FS (Fig. 3(A) to (E)), while comparisons between all ingredients are displayed in online Supplementary Table S1.

Beta-2-microglobulin (*B2M*), espin (*ESPN*), hydroxymethylbilane synthase (*HBMS*), interferon beta (*IFNβ*), *IL1β* and proliferating cell nuclear antigen (*PCNA*) genes showed low

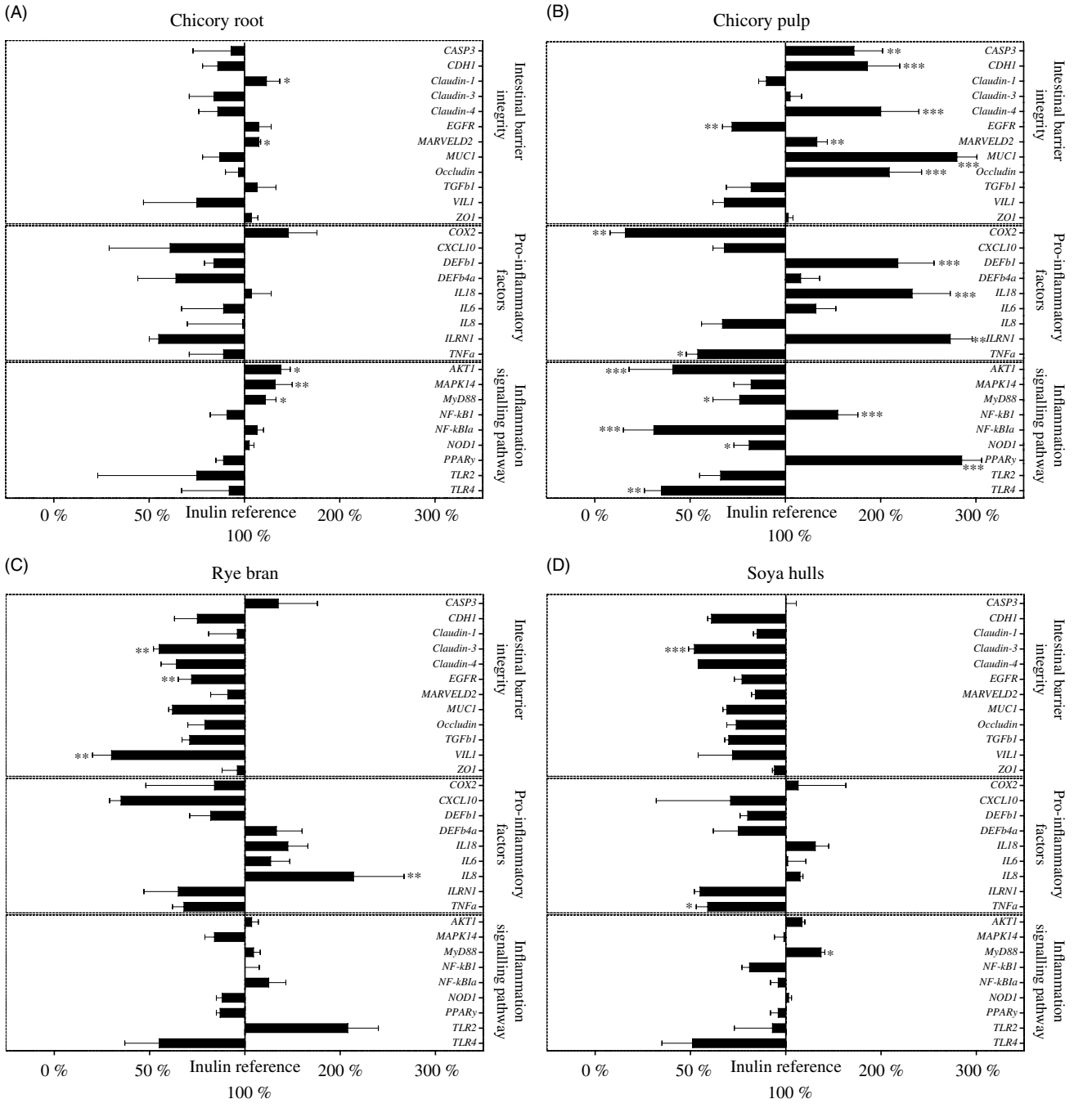

Fig. 3. Impact of fermentation supernatant (FS) 10 % (v/v) collected after 12 h on gene expression in intestinal porcine epithelial cells (IPEC-J2). (A) Chicory root; (B) chicory pulp; (C) rye bran; (D) soya hulls; (E) citrus pulp. Values are means of triplicate well-measurements with their standard errors of the mean. Gene expression was not stable between the control treatment and the 0·8-µm ø FS treatments for eleven reference genes studied; hence, the different fermented ingredients were compared with inulin. Figures display the % of difference of the different genes for one ingredient in comparison with inulin, considered as 100 %. Significantly different from inulin FS: *, **, *** for false discovery rate corrected-P <0·5, <0·01 and <0·0001, respectively. The geometric mean of ribosomal protein L 13a (*RPL13a*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), peptidylprolyl isomerase A (*PPIA*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) was used to normalise samples. *AKT1*, serine/threonine-protein kinase 1; *MAPK14*, mitogen-activated protein kinase 14; *MyD88*, myeloid differentiation primary response 88; *NF-kBIα*, NF-κB inhibitor alpha; *NOD1*, nucleotide-binding oligomerisation domain-containing protein 1; *TLR*, toll-like receptor; *CCL5*, chemokine ligand 5; *COX2*, cyclo-oxygenase 2; *CXCL10*, C-X-C motif chemokine 10; *DEFβ*, defensin beta; *EGFR*, epidermal growth factor receptor; *IFN*, interferon; *ILRN1*, IL-1 receptor antagonist; *MCP1*, monocyte chemoattractant protein 1; *CASP3*, caspase 3; *CDH1*, E-cadherin; *MARVELD2*, tricellulin; *MUC1*, mucin 1; *TGFβ1*, transforming growth factor beta 1; *VIL1*, villin 1; *ZO-1*, zonula occludens-1.

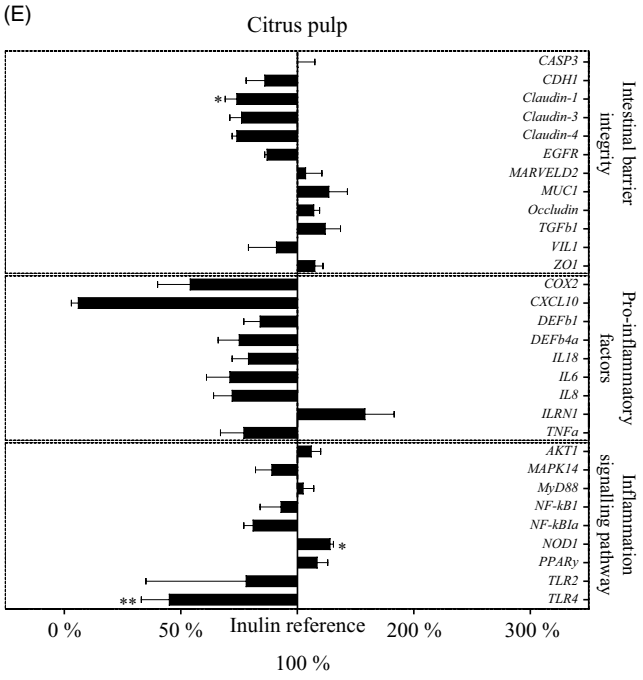

Fig. 3. (Continued).

expressions in IPEC-J2 cells. Chemokine ligand 5 (*CCL5*) and monocyte chemoattractant protein 1 (*MCP1*) primers' efficiencies did not range between 90 and 110 %, and their results were excluded from the study.

The mRNA levels of all target genes were similar between chicory root and inulin except for *AKT1*, mitogen-activated protein kinase 14 (*MAPK14*), myeloid differentiation primary response 88 (*MyD88*), claudin-1 and *MARVELD2* gene expressions which were significantly higher in chicory root (Fig. 3(A)). Chicory pulp displayed higher adherens (*CDH1*, i.e. e-cadherin) and tight junction gene expression levels (occludin, claudin-4 and *MARVELD2*, i.e. tricellulin) in comparison with inulin ($P < 0{\cdot}01$; Fig. 3(B)). Mucin 1 mRNA (*MUC1*) and caspase 3 (*CASP3*) levels were also higher for chicory pulp than for inulin, whereas epidermial growth factor receptor (*EGFR*) levels were down-regulated for chicory pulp. Considering inflammatory pathways, serine/threonine-protein kinase 1 (*AKT1*), cyclo-oxygenase 2 (*COX2*), NF-κB inhibitor alpha (*NF-kBIα*), nucleotide-binding oligomerisation domain-containing protein 1 (*NOD1*), toll-like receptor 4 (*TLR4*) and *TNFα* gene expression levels were significantly lower in chicory pulp compared with inulin, whereas the opposite was observed for the genes defensin beta 1 (*DEFβ1*), *NF-kB1*, *IL18*, IL-1 receptor antagonist (*ILRN1*) and *PPARγ*. Rye bran exhibited significantly lower vilin 1 (*VIL1*), claudin-3 and *EGFR* compared with inulin and higher gene expression levels of *IL8* (Fig. 3(C)). *MyD88* mRNA levels were significantly up-regulated in soya hulls compared with inulin, while *TNFα* and claudin-3 were down-regulated ($P < 0{\cdot}05$; Fig. 3(D)). *NOD1* gene expression levels were significantly up-regulated in citrus pulp compared with inulin, while *TLR4* and claudin-1 were down-regulated (Fig. 3(E)).

Discussion

The first aim of the present research was to determine if fibre-rich agricultural and industrial by-products reach the same prebiotic potential as inulin in terms of gas and SCFA productions and microbiota profiles. Inulin and chicory root exhibited fast and intense fermentation patterns with the highest cumulative gas productions (A) and rate of fermentation (R_{MAX}) and the lowest time to half asymptote (B) and time to reach R_{MAX} (T_{MAX}). Chicory root, with a higher ratio of soluble to insoluble polysaccharides and the soluble fraction being mainly composed of fructans, is highly fermentable and therefore is more likely to be fermented at the end of the small intestine[55]. Chicory pulp displayed slower fermentation kinetics which is related to its high insoluble fibre content (high NDF and ADF levels) and its low amount in fructans, mainly composed of polysaccharides with high polymerisation degree[30]. This supports the hypothesis that the rapidity and extensiveness of the fermentation are modulated by the fructan content of the ingredient which was already demonstrated by Shim *et al.*[56] and Pellikaan *et al.*[57] and previous experiments[30].

Similarly to chicory pulp, citrus pulp demonstrated mid-range values for gas kinetics (intermediate values for A, B and R_{MAX}) due to a mixture of soluble (highly methylated pectin) and insoluble fibres (cellulose) with the soluble fraction being fermented rapidly, while the insoluble one is extensively fermented following the disappearance of the soluble fibres[58].

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Rye bran is composed of considerable amounts of fructan, although lower to the chicory by-products. Nevertheless, the complexity of the carbohydrate fraction[59] rich in insoluble fibres, in particular water-unextractable arabinoxylans, presumably accounts for the intermediate rate of fermentation (R_{MAX}) and the low final gas production (A) for this ingredient in comparison with the fructan-based ingredients. The same effect can be observed with soya hulls characterised by high concentrations of cellulose (high ADF) which is known to ferment to a lower extent. These ingredients are more likely to be fermented in the hindgut.

After 24 h of fermentation, chicory root and pulp produced significant amounts of total SCFA, like inulin, due to their amount in fructo-oligosaccharides. The high butyric acid content induced by the fermentation of fructan-rich ingredients, that is, chicory root and inulin, was already reported in porcine studies[60] and is confirmed in the present study. Total SCFA and butyrate amounts are highly correlated not only with an extensive fermentation but also with the fructan content[30], which is shown by similar results in terms of gas production, total SCFA and butyrate ratios for inulin and chicory root. Similarly, no remarkable difference was reported in terms of microbiota populations between the two ingredients. Surprisingly, the bifidogenic capacities of fructo-oligosaccharides reported in the literature, in humans[61,62], are not apparent in our results. The consumption of fructan-based ingredients provides acetic acid to butyrate-producing bacteria used as a co-ingredient to produce butyrate which corroborates with the low acetate and high butyrate levels in inulin and chicory root at 24 h. Contradictory, inulin and chicory root displayed the lowest level of *Lactobacillus* spp. and the highest levels of lactate after 6 and 12 h of fermentation. This can be explained by the fact that the mucin carriers, acting as adhesive sites for *Lactobacillus* spp., are more representative of the genus abundance than the fermentation broth[39].

Acetate productions for chicory pulp, citrus pulp and soya hulls were higher compared with the ratio of inulin, probably due to the high concentrations in cellulose, and the moderate soluble fibre and hemicelluloses contents. Remarkably, citrus pulp showed the highest butyrate producing capacity, based upon the *Clostridium* cluster IV (along with chicory pulp and soya hulls) and *Clostridium* cluster XIVa results, while chicory root and inulin were having low relative levels of these two clusters.

Our study highlighted that rye bran induced the greatest stimulation of *Bifidobacterium* spp. at 12 and 24 h and the second greatest amount of butyryl-CoA:acetate-CoA transferase after 24 h. Fermentation of arabinoxylan was associated with a proliferation of *Bifidobacterium* spp., in several trials with human feces[63–65] which is in line with our results.

The second aim of the research was to compare the immunomodulatory profiles of the five ingredients FS compared with inulin FS on cultured IPEC-J2. The findings in the present study showed that chicory pulp was able to increase gene expression levels of tight and adherens junctions thereby enhancing the barrier function of intestinal epithelium with higher *CDH1*, occludin, claudin-4 and tricellulin levels in comparison with inulin. Inulin FS had been shown to increase TEER in human cell models, therefore reinforcing gut barrier tightness[66,67] which is in line with our findings. The high-throughput qPCR study supported the idea that complete FS may modulate the inflammatory state of the intestinal epithelial layer. Our results indicate that chicory pulp FS exerts anti-inflammatory effects on IPEC-J2 that mainly depend on the *TLR* as well as the *NOD* signalling pathways, both related to bacterial pattern recognition and ligation[68,69]. In our study, chicory pulp triggered the expression of *PPARγ* and inhibited pro-inflammatory cytokines such as *TNFα*. *PPARγ* was reported to inhibit the production of inflammatory cytokines in different cell types by interfering with *TLR*-dependent signalling pathway[20,70,71], which is in agreement with the present study. Furthermore, chicory pulp might have increased the transcription activity of pro-apoptotic targets via the *PI3K-AKT* pathway, seen by the decrease in *AKT1* gene expression level, leading to the inhibition of pro-survival target genes[72] and an up-regulated caspase-3 mRNA levels, a marker of advanced apoptosis[73]. As intermediate levels of butyrate were found during the fermentation of chicory pulp in comparison with inulin, it seems that a direct effect of the ingredient or a synergistic effect of the ingredients with fermentation metabolites and/or microbiota is responsible for the reinforcement in barrier function and the anti-inflammatory effect. Besides, bacterial modulation was found for this ingredient with a remarkable increase in *Clostridium* clusters IV and XIVa bacteria in comparison with inulin which might also affect the epithelial barrier tightness and inflammatory response[8].

The gene expression levels of all tight and adherens junctions target genes were similar between chicory root and inulin except for claudin-1 and tricellulin mRNA levels which were significantly higher in chicory root. This is in line with Pham *et al.*[67] who found that inulin and dried chicory root FS played a protective role in reversing the gut permeability, probably due to butyrate, in HT29-MTX and HT29 cell models. Chicory root showed a significant induction of genes involved in the *MAPK* signalling pathway (*AKT1*, *MAPK14*, *NF-kB* and *MyD88*) typically resulting in up-regulation of different pro-inflammatory cytokines[74,75], although no up-regulation of pro-inflammatory cytokines (*IL6*, *IL8* and *IL18*) was induced by the chicory root supernatant. It might be that the signalling pathway did not reach the end-target cytokines after 24 h of FS exposure on the IPEC-J2. Rye bran, citrus pulp and soya hulls showed no additional immune-related activities in comparison with inulin in IPEC-J2. The increased *Bifidobacterium* spp. levels due to the fermentation of rye bran or the proliferation in butyrogenic species arising from the fermentation of soya hulls and citrus pulp did not induce differential gene expressions in IPEC-J2.

One limitation of our study was the use of supernatants collected from a batch fermentation model with substrate depletion, pH reduction and the accumulation of metabolites as major drawbacks of the *in vitro* technique. Moreover, the different FS could not be compared with the sham-treated cells, due to the instability of the reference genes between treatments. This implies that the addition of FS highly impacted the cell regulatory function which remains one limitation of our model.

Seen the positive effects of chicory by-products on gene expression related to gut barrier, industrial and agricultural by-products such as chicory root and pulp may be an interesting ingredient to be further tested in the feed of piglets in the weaning period, to modulate intestinal fermentation and consequently gut immunity and the mucosal barrier integrity. Then, *in vivo* trials should further evaluate the dosage of inclusion in the diet[76].

In conclusion, chicory root reached the same prebiotic potential as inulin in terms of fermentation kinetics and metabolites production, while soya hulls, rye bran and citrus pulp positively modulated health-promoting microbiota populations. We have also assessed that chicory pulp complete FS promoted the intestinal barrier integrity as can be seen by the up-regulated expression of tight and adherens junction genes in comparison with inulin. Chicory pulp seemed to induce different immunomodulatory pathways such as anti-inflammatory and pro-apoptotic regulations.

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Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0007114519002873

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