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Differential effects of inulin or its fermentation metabolites on gut barrier and immune function of porcine intestinal epithelial cells

Julie Uerlings[a,c], Martine Schroyen[a], Els Willems[b], Sofie Tanghe[b], Geert Bruggeman[b], Jérôme Bindelle[a], Nadia Everaert[a,*]

[a] Precision Livestock and Nutrition Unit, TERRA Teaching and Research Centre, Gembloux Agro-Bio Tech, ULiège, Gembloux, Belgium
[b] Royal Agrifirm Group, Apeldoorn, the Netherlands
[c] Research Foundation for Industry and Agriculture, National Scientific Research Foundation (FRIA-FNRS), Brussels, Belgium

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ABSTRACT

Prebiotics can modulate gut fermentation and improve intestinal barrier function in mammals. First, inulin fermentation profile was tested in a three-step *in vitro* model of the piglet's gastro-intestinal tract combining a hydrolysis – dialysis step to a batch fermentation. Then, the differential effects of digested inulin (after the hydrolysis – dialysis steps) or fermented inulin (after the fermentation step) on the expression of gut barrier and immune-related genes of IPEC-J2 cells were investigated by high-throughput qPCR. Inulin was associated with elevated short-chain fatty acids and butyrate levels. Upregulated expressions of tight and adherens junction genes were observed in IPEC-J2 cells supplemented with inulin fermentation supernatant compared to control IPEC-J2 cells and digested inulin. Therefore, metabolites arising from the fermentation process, including butyrate, could be responsible for the reinforcement of the barrier function.

1. Introduction

To date, prebiotics represent a wide-spread dietary approach to modulate intestinal fermentation and manipulate gut ecology for health purposes (Roberfroid, 2007). The most commonly used prebiotics, in humans (Roberfroid, Van Loo, & Gibson, 1998) and pigs (Samanta, Jayapal, Senani, Kolte, & Sridhar, 2013) diets, are fructo-oligosaccharides such as inulin. Prebiotics help creating and maintaining an optimal environment in the host gastro-intestinal tract by selectively stimulating the proliferation and metabolic activity of health-associated microbiota communities and lowering the pathogenic bacteria load (Gibson & Roberfroid, 1995). The fermentation of prebiotics by the endogenous microbiota yields short-chain fatty acid (SCFA) end-products, acetate, propionate, and butyrate. The latter is of special interest for its anti-inflammatory properties (Canani et al., 2011) and is used as energy source by the colonocytes (Roediger, 1982), leading to a reinforcement of the intestinal barrier integrity (Peng, Li, Green, Holzman, & Lin, 2009). Moreover, a reduction of butyrate and butyrate-producing bacteria has been related to colonic-derived diseases such as inflammatory bowel disease (Canani et al., 2011) and Crohn's disease (Sokol et al., 2008). Alternatively, acetate and propionate may influence cholesterol metabolism (Chen, Anderson, & Jennings, 1984) and appetite regulation (Chambers, Morrison, & Frost, 2015; Frost et al., 2014), respectively. The ratio between individual SCFA and the total amount of SCFAs produced vary with the source of prebiotic (Grootaert et al., 2009; Van den Abbeele, Venema, Van de Wiele, Verstraete, & Possemiers, 2013).

There is increasing evidence of a strong interaction between the intestinal barrier mucosa, the gut microbiota and the local immune system (Takiishi, Fenero, & Câmara, 2017). The intestinal mucosa, with lined-up enterocytes joined by tight and adherens junctions, together with a mucus layer, creates a physical barrier allowing the absorption of nutrients while preventing bacterial translocation (Ulluwishewa et al., 2011). This dynamic structure is constantly remodelled due to interactions with feed residues, metabolites, as well as with pathogenic and commensal bacteria. This way, a compromised barrier integrity could trigger an exacerbated inflammatory response. Alternatively, the use of prebiotics to preserve the intestinal mucosal barrier function has gained considerable interest in the recent years and inulin has been postulated to enhance gut barrier integrity *in vitro* (Van den Abbeele et al., 2018) and *in vivo* (Russo et al., 2012) in humans.

So far, many studies focused on the action of prebiotics on the intestinal barrier mucosa via direct signalling routes initiated by the oligosaccharides themselves, and much less by their bacterial

* Corresponding author at: Gembloux Agro-Bio Tech, Passage des Déportés, 2, 5030, Gembloux, Belgium.
E-mail address: nadia.everaert@uliege.be (N. Everaert).

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metabolites. Several mechanisms have been attributed to this direct effect on gut health, such as interference with pathogenic attachment (Sun, Gänzle, & Wu, 2019), interaction with recognition molecules (Shoaf, Mulvey, Armstrong, & Hutkins, 2006) and influence on cytokine transduction pathways (Ortega-González et al., 2014; Zenhom et al., 2011). However, studies conducted with single bioactive compounds do not take into account the fermentation process that may affect the physiochemical properties of the ingredient and the possible synergistic activities between metabolites (Nielsen et al., 2018). Many *in vitro* models have been investigating the host-prebiotic or the host-metabolite interactions separately. Detailed evidence is still missing on which health-beneficial effects should be attributed to the direct and/or the prebiotic effects of dietary fibre ingredients in the intestine. Owing to the complexity of the intestinal chyme and lumen, it is important to implement a mechanistic, although holistic approach combining an *in vitro* fermentation model of the gastro-intestinal tract with intestinal epithelial cell cultures, studying the effect of bioactive compounds on cell lines. In this study, the intestinal epithelial cell line (IPEC-J2), of porcine origin, was chosen to represent the intestinal wall in the *in vitro* model of the young piglet's gastro-intestinal tract. Although the IPEC-J2 cell line is derived from the small intestine of young piglets, no colonic cell line from porcine origin is available. Moreover, the *in vitro* batch fermentation was prepared with feces of young piglets which is a suitable and representative inoculum to mimic the *in vivo* gut fermentation (Williams, Voigt, & Verstegen, 1998) and justifies the model chosen for the following research.

The aim of the study was to evaluate the prebiotic potential of inulin to modulate intestinal fermentation for young mammalian health purposes and to investigate if inulin digesta (inulin DI) or inulin fermentation metabolites (inulin FS) induced differential effects on intestinal immunity and barrier function using IPEC-J2 gene expression responses.

2. Materials and methods

2.1. Analysis of inulin

Inulin (Fibruline Instant) was provided by Cosucra Warcoing SA (Belgium). It was analyzed for organic matter (AOAC 923.03), dry matter (AOAC 967.03), crude protein (Foss Kjeltec Analyzer Unit 2300, Hilleroed, Denmark; CP = N × 6.25), fat content (Soxhlet method; AOAC 920.29) and neutral (NDF) and acid (ADF) detergent fibre (Foss Fibrecap system, Hilleroed, Denmark; Van Soest, Robertson, and Lewis (1991)). Non-cellulosic total monosaccharides composition was determined according to the method of Englyst and Cummings (1984) adapted by Aguedo, Fougnies, Dermience, and Richel (2014). The fructan molecular mass distribution was assessed by size-exclusion high performance liquid chromatography (HPSEC) performed with a Waters 2690 Alliance chromatograph (Waters, Milford, USA) coupled to a refractometer Waters 2410 as previously described (Aguedo et al., 2014).

2.2. In vitro digestion and batch fermentation of inulin

Inulin was studied using a modified three-step *in vitro* model of the pig's gastro-intestinal tract (Bindelle, Buldgen, Boudry, & Leterme, 2007) combining an enzymatic hydrolysis and dialysis to a batch fermentation with fecal microbiota (Uerlings et al., 2019a).

For the batch fermentation, a fecal inoculum was prepared from a buffer solution composed of salts and minerals (pH 6.8; Menke and Steingass (1988)) devoid of reducing agent (Poelaert et al., 2018) and frozen feces (2.5% [w/v]) from piglets, under anaerobic conditions (Invivo$_2$, Led Techno, Heusden-Zolder, Belgium). Feces were previously collected from pre-weaned three week-old-piglets (male and female) by fecal stimulation with sterile swabs. All experimental procedures led on piglets (feces 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). They were carried out in accordance with the U.K. Animals Act, 1986 and EU Directive 2010/63/EU for animal experiments.

Three mucin microcosms (Tran et al., 2016) were added to each fermentation vial to enhance the growth of communities found on mucus, with inulin digesta or not (blank vials). The vials (n = 3 for the inulin or blank treatments per time-point) were placed into an agitating water-bath at 39 °C with 50 rpm agitation and the fermentation supernatants were stored at −80 °C.

2.3. Fermentation kinetics profile of the in vitro batch fermentation

The released gas volumes (n = 3 vials for the inulin or blank treatments) were repeatedly recorded with a Tracker 200 manometer (Bailey & Mackey Ltd, Birmingham, UK) at following time points; 2, 5, 8, 12, 16, 20, 24, 48 and 72 h according to the model of Groot, Cone, Williams, Debersaques, and Lantinga (1996) and gas production recordings were fitted to the mathematical monophasic model, 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}, the time to reach R_{MAX}.

2.4. Short-chain fatty acid profile of supernatants from in vitro batch fermentation

Fermentation broths sampled after 6, 12 and 24 h of fermentation (n = 3 vials for the inulin or blank treatments) were analyzed by isocratic high-performance liquid chromatography (HPLC) using the Alliance System e2695 (Waters, Milford, CT) with an Aminex HPx-87H column (BioRad, Hercules, CA) as previously described (Uerlings et al., 2019a).

2.5. Inulin digesta (DI) preparation

Inulin digesta (inulin DI), pooled from 8 experimental units that had undergone the *in vitro* hydrolysis and dialysis steps, was diluted using sterile PBS to reach 0.5% [w/v] as final concentration. Then, the suspension was sonicated in a water bath (3 times of 30 s each) and centrifuged (460 g, 5 min) as described by Becker, Galletti, Roubos-van den Hil, and Van Wikselaar (2007). Inulin DI was frozen at − 20 °C until further application on IPEC-J2 cells.

2.6. Fermentation supernatant (FS) preparation

Inulin fermentation broths (inulin FS) from 3 different fermentation vials as well as fermentation broth from 3 blank fermentation vials (blank FS) were pooled after 12 h of fermentation, were sterile-filtered with 0.22-µm pore filters and were stored at −80 °C until further application on IPEC-J2 cells.

2.7. IPEC-J2 cell line and culture conditions

IPEC-J2 cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in complete Dulbecco's Modified Eagle 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 selenium (all from Sigma, Saint Louis, MO). Culture medium was renewed once every two days, and cells were passaged when they reached confluence.

2.8. Measuring IPEC-J2 cell viability

The viability test (n = 6 culture well-replicates per treatment) was used to determine the concentrations of inulin DI, inulin FS and blank

FS suitable to use in the gene expression study. Cell proliferation was measured with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. IPEC-J2 cells between passages 15 and 20 were seeded in 96-well flat bottomed plates at a density of 20 000 cells / 100 µL (100 µL per 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 blank FS and inulin FS (2.5, 5, 10, 15, 50% [v/v]) or inulin DI (0.25, 0.5, 0.75, 1, 1.25, 2.50% [w/v] (Becker et al., 2007)). After incubation with different concentrations of supernatants for 24 h, the culture medium was removed. Next, fresh antibiotic-free culture medium and 15 µL of MTT reagent (Promega, Madison, WI) were added to each well for another 4 h at 37 °C prior to measurement of cell viability. The absorbance was determined at 570 nm in a micro-plate reader (VICTOR plate reader, PerkinElmer, Waltham, MA). According to the cell viability test and to the literature using a similar methodology (Borowicki et al., 2010; Fässler, Gill, Arrigoni, Rowland, & Amadò, 2007; Stein, Borowicki, Scharlau, & Glei, 2010), blank FS, inulin FS and inulin DI were applied at 10% [v/v] for the FS and 0.5% [w/v] for the DI.

2.9. Expression of barrier function and immune-related genes in IPEC-J2

IPEC-J2 cells between passages 15 and 20 were seeded in 24-well plates at a density of 2.5×10^5 cells / mL (1 mL per well). Prior to the treatment, confluent monolayers of the IPEC-J2 cells were washed with plain medium without antibiotics. Blank FS, inulin FS and inulin DI were applied at 10% [v/v] for the FS and 0.5% [w/v] for the DI for 24 h (n = 3 culture well-replicates per treatment). For sham-stimulation, cells were maintained in the culture medium for 24 h (n = 3).

Total RNA from IPEC-J2 cells treated with the blank FS, inulin FS, inulin DI and control cells was extracted using the RNeasy Mini kit (RNeasy Mini Kit, Qiagen, Hilden, Germany) as previously described (Uerlings et al., 2019b). Extracted RNA was converted into cDNA by reverse transcription using Reverse Transcription Master Mix (Fluidigm Corporation, San Francisco, CA). High-throughput qPCR was performed as previously described (Uerlings et al., 2019b) with intron spanning primer pairs (Table 1). High-throughput qPCR was performed in 48x48 dynamic array integrated fluidic circuits (Fluidigm Corporation, San Francisco, CA). After loading, the dynamic array was placed in BioMark HD Real-Time PCR System (Fluidigm Corporation, South San Francisco, CA), and the following cycle parameters were used: 60 s at 95 °C, followed by 35 cycles (5 s at 96 °C and 20 s at 60 °C).

Quantification cycles (*Cq*) were acquired using the Fluidigm real-time PCR analysis software 3.0.2 (Fluidigm Corporation, San Francisco, CA). The geometric mean of four reference genes (ribosomal protein L13a (*RPL13a*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), peptidylprolyl isomerase A (*PPIA*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*)) stably expressed between treatments using NormFinder (Andersen, Jensen, & Ørntoft, 2004) was used to normalize samples. For each target, the relative expression level was calculated by the $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen, 2001).

2.10. Statistical analysis

Homogeneity between variances and normality among treatments was confirmed using Bartlett's and Ryan-Joiner's tests, respectively. The experimental unit for SCFA analysis was the fermentation vial and the one for the gene expression assay was the cell culture well. The experimental data were subjected to GLM procedures and the comparison of means was evaluated by post-hoc Tukey's multiple range HSD using SAS 9.4 software (SAS Institute) with one fixed criteria of classification for the gene expression assay (type of treatment). The analyses of SCFA 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 profiles, when a significant interaction was found, parameters were studied by one-way ANOVA per time point. Adjusted p-values for the gene expression assay were obtained using a false discovery rate (FDR) correction with the linear method of Benjamini and Hochberg. P-values < 0.05, < 0.01 and < 0.001 were considered as statistically significant, highly significant and very highly significant.

3. Results

3.1. Inulin and its fermentation kinetics

Inulin contained high amounts of fructans (89.4%; Table 2) and was characterized by a weight average molecular weight (Mw) of 5690 Da (Fig. 1). The ingredient induced a total gas production of 300 mL g^{-1} DM (A) and a maximal rate of fermentation of 27.4 mL g^{-1} DM h^{-1} (R_{MAX}). The time to reach R_{MAX} was 4.4 h (T_{MAX}) and the half-time to asymptotic gas production was 7.3 h (B) as displayed in figure S1. The interaction between the treatments and the time of fermentation was significant for all the measured metabolites except for lactate and total SCFA amounts (Fig. 2A and B). After 12 and 24 h, fermentation of inulin induced greater molar ratio of propionate and butyrate in comparison to the blank vials ($P < 0.001$; Fig. 2C) which consequently showed higher acetate proportions.

3.2. IPEC-J2 cell viability

In order to choose the most appropriate concentrations of fermentation supernatants and digesta for the IPEC-J2 model, a cell viability assay was conducted (figure S2A and S2B). Inulin DI induced a reduction of the cell viability under 50% with a concentration superior to 0.75% [w/v] (figure S2A) and 0.5% [w/v] was chosen as the concentration for the immunomodulatory model. Inulin and blank FS collected after 12 h of fermentation were not toxic for IPEC-J2 at a concentration lower than 25% [v/v] with a reduction of the cell viability around 50% at the cited concentration (EC50). A concentration of 10% [v/v] led to a reduction of approximately 30% of cell viability for both supernatants (figure S2B). According to these results, 10% [v/v] was chosen as FS concentration for the immunomodulatory model.

3.3. Expression of barrier function and immune-related genes in IPEC-J2

Beta-2-microglobulin (*B2M*), espin (*ESPN*), hydroxymethylbilane synthase (*HBMS*), interferon beta *(IFNβ)*, interleukin 1-beta (*IL1β*) and proliferating cell nuclear antigen (*PCNA*) genes showed low expressions in the high-throughput qPCR and were therefore undetectable. 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.

Concerning the inflammation signalling pathways and the pro-inflammatory proteins (Fig. 3A and B), the mRNA levels of all 18 target genes were similar between inulin DI and the control cells except for the serine/threonine-protein kinase 1 (*AKT1*) and the C-X-C motif chemokine 10 (*CXCL10*) which were significantly higher and the nuclear factor-kappa B inhibitor alpha (*NF-kBIα*) and the tumor necrosis factor alpha (*TNFα*) which were significantly lower for inulin DI compared to the control cells ($P < 0.001$; Fig. 3A and B). The mitogen-activated protein kinase 14 (*MAPK14*), myeloid differentiation primary response 88 (*MyD88*), nucleotide-binding oligomerization domain-containing protein 1 (*NOD1*), defensin beta 1 (*DEFβ1*), defensin beta 4a (*DEFβ4a*), interleukin-18 (*IL18*), *TNFα* and the peroxisome proliferator-activated receptor gamma (*PPARγ*) levels were significantly higher for inulin FS in comparison to inulin DI and the opposite was observed for *AKT1*, *CXCL10* and the cyclooxygenase 2 (*COX2*). *AKT1, COX2* and *CXCL10* levels also differed between inulin DI and both the inulin FS and blank FS with the digesta displaying the highest levels.

Both inulin FS and blank FS induced elevated *NOD1*, *MAPK14,*

Table 1
Nucleotide sequences of primers for the IPEC-J2 cell response.

Target family	Target gene		Primer sequence 5′ → 3′	Accession number
Housekeeping genes	*ACTB*	F	CTACGTCGCCCTGGACTTC	XM_003124280.5
		R	GCAGCTCGTAGCTCTTCTCC	
	B2M	F	ACCACTTTTCACACCGCTC	NM_213978.1
		R	GCTTTCCGTTTTCCGCTGG	
	ESPN	F	CACTGGCAAAGTGAGAGTCCT	XM_021095253.1
		R	TGTGGTCAGCCCCTTACTCT	
	GAPDH	F	GATGGTGAAGGTCGGAGTGAA	XM_021091114.1
		R	GTGGAGGTCAATGAAGGGGT	
	HBMS	F	CCTTTAGCGGGGGAAATCAC	XM_021102144.1
		R	CTGAAGCCCATCCCAGCTAA	
	HPRT1	F	AATTCTTTGCTGACCTGCTGGA	XM_021079503.1
		R	TCCACCAATTACTTTTATATCGCCC	
	PCNA	F	CTGCAAGTGGAGAACTCGGAA	NM_001291925.1
		R	AAGTTCAGGTACCTCAGTGCAA	
	PPIA	F	GGGACCTGGAAACCAAGAAGTG	XM_013985800.2
		R	ACTTTGTCTGCAAACAGCTCCAATC	
	RPL13a	F	ATTGTGGCCAAGCAGGTACT	XM_013998640.2
		R	AATTGCCAGAAATGTTGATGC	
	RPL32	F	GCTTGAAGTGCTGCTAATGTG	XM_021068582.1
		R	GGATTGGTGACCCTGATGGC	
	RPL4	F	GAGAAACCGTCGCCGAATCC	XM_005659862.3
		R	CCCACCAGGAGCAAGTTTCAA	
	SDHA	F	GTCGTCGGCCAAAGTTTCAG	XM_021076930.1
		R	TGTGTTAAACCCGGCCTCAG	
	TBP	F	CGGACCACCGCACTGATATT	XM_021085483.1
		R	TTCTTCACTCTTGGCTCCCG	
	YWHAZ	F	TTGTAGGAGCCCGTAGGTCA	NM_001315726.1
		R	AGCACCTTCCGTCTTTTGCT	
Inflammation signalling pathway genes	*AKT1*	F	CTAAGCCCAAACACCGCGT	XM_021081499.1
		R	TCAGGATCTTCATGGCGTAGT	
	MAPK14	F	TACCCGAGCGTTACCAGAAC	XM_001929490.6
		R	TTCACTGCAACACGTAACCCA	
	MyD88	F	GCATCACCATTCGAGATGACC	NM_001099923.1
		R	TCCTGCACAAACTGGGTATCG	
	NF-kB1	F	AAGAAGTCCTACCCTCAGGTCA	NM_001048232.1
		R	CAGTGACAGTGCAGATCCCA	
	NF-kBIα	F	GAGGATGAGCTGCCCTATGAC	NM_001005150.1
		R	CCATGGTCTTTTAGACACTTTCC	
	NOD1	F	GTCGTCAACACCGATCCAGT	NM_001114277.1
		R	CCTCCTTCTGGGCATAGCAC	
	PPARγ	F	ACAGCGACCTGGCGATATTTA	XM_005669784.3
		R	GAGGACTCTGGGTGGTTCAA	
	TLR2	F	GTTTTACGGAAATTGTGAAACTG	XM_005653576.3
		R	TCCACATTACCGAGGGATTT	
	TLR4	F	ATGATTCCTCGCATCCGCCT	NM_001113039.2
		R	AATTCAGCTCCATGCATTGGTAA	
Pro-inflammatory genes	*CCL5*	F	ACACCACACCCTGCTGTTTT	NM_001129946.1
		R	TCTTCTCTGGGTTGGCACAC	
	COX2	F	TCGAGATGATCTACCCGCCT	NM_214321.1
		R	ACATCATCAGACCAGGCACC	
	CXCL10	F	CCCACATGTTGAGATCATTGC	NM_001008691.1
		R	GCTTCTCTCTGTGTTCGAGGA	
	DEFβ1	F	TTCCTCCTCATGGTCCTGTTAC	MF925344.1
		R	CCACAGGTGCCGATCTGTTTC	
	DEFβ4a	F	CAGGATTGAAGGGACCTGTT	NM_214442.2
		R	CTTCACTTGGCCTGTGTGTC	
	IFNβ	F	TTCGAGGTCCCTGAGGAGATT	NM_001003923.1
		R	GCTGGAGCATCTCGTGGATAA	
	IL1β	F	CCAAAGAGGGACATGGAGAA	XM_021085847.1
		R	GGGCTTTTGTTCTGCTTGAG	
	IL18	F	CTGAAAACGATGAAGACCTGGA	XM_005667326.2
		R	CCTCAAACACGGCTTGATGTC	
	IL6	F	TGGGTTCAATCAGGAGACCT	NM_001252429.1
		R	CAGCCTCGACATTTCCCTTA	
	IL8	F	GACTTCCAAACTGGCTGTTGC	JF906514.1
		R	ATTTGGGGTGGAAAGGTGTG	
	ILRN1	F	TGCCTGTCCTGTGTCAAGTC	NM_214262.1
		R	GTCCTGCTCGCTGTTCTTTC	
	MCP1	F	CTCACTGCAGCCACCTTCT	NM_214214.1
		R	CACTTGCTGCTGGTGACTCT	
	TNFα	F	TCTGCCTACTGCACTTCGAG	NM_214022.1
		R	GTTGATGCTCAAGGGGCCA	

(*continued on next page*)

Table 1 (*continued*)

Target family	Target gene		Primer sequence 5′ → 3′	Accession number
Intestinal barrier integrity genes	*CASP3*	F	AAGCAAATCAATGGACTCTGGAA	NM_214131.1
		R	TTGCAGCATCCACATCTGTACC	
	CDH1	F	AGCCCTGCAATCCTGGCTTT	NM_001163060.1
		R	AGAAACATAGACCGTCCTTGGC	
	Claudin-1	F	GGTGACAACATTGTGACGGC	NM_001244539.1
		R	TACCATCAAGGCACGGGTTG	
	Claudin-3	F	TATCACAGCGCGGATCACC	NM_001160075.1
		R	CTCTGCACCACGCAGTTCAT	
	Claudin-4	F	CTTCATCGGCAGCAACATCG	XM_013995522.2
		R	CGAGTCGTACACCTTGCACT	
	EGFR	F	GCACAAGGACAACATCGGCTC	NM_214007.1
		R	GATCTTGACATGCTGCGGTGT	
	MARVELD2	F	CTCAGCCCCGCCATTACCTG	NM_001243948.1
		R	TAGAGGTGATGTGCTGTTGCC	
	MUC1	F	GGATTTCTGAATTGTTTTTGCAG	XM_021089728.1
		R	ACTGTCTTGGAAGGCCAGAA	
	Occludin	F	AACGTATTTATGACGAGCAGCCC	NM_001163647.2
		R	CACTTTCCCGTTGGACGAGTA	
	TGFβ1	F	CATTCACGGCATGAACCGGC	XM_021093503.1
		R	CGCACGCAGCAGTTCTTCTC	
	VIL1	F	ACAAAGGTCGCTGTCCTCCA	XM_001925167.6
		R	TGACCTGGGCGTTCAGTTTG	
	ZO-1	F	AAGGTCTGCCGAGACAACAG	XM_021098827.1
		R	TCACAGTGTGGTAAGCGCAG	

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

Table 2
Chemical composition (g/kg dry matter) of inulin constituent monosaccharides composition of the non-cellulosic polysaccharide fraction.

Inulin	
Dry matter (g/kg)	953
Fat (g/kg DM)	–
Protein (g/kg DM)	–
Ash (g/kg DM)	0.7
NDF[a] (g/kg DM)	4.3
ADF[b] (g/kg DM)	1.2
Fructan (g/kg DM)	894.2
CHO composition	
Rhamnose (g/kg DM)	4.8
Arabinose (g/kg DM)	2.3
Xylose (g/kg DM)	1.8
Mannose (g/kg DM)	95.4
Glucose (g/kg DM)	162.2
Galactose (g/kg DM)	7.7

Abbreviations: acid detergent fibre (ADF); carbohydrates (CHO); dry matter (DM); neutral detergent fibre (NDF).
[a] NDF : hemicelluloses + cellulose + lignin
[b] ADF : cellulose + lignin

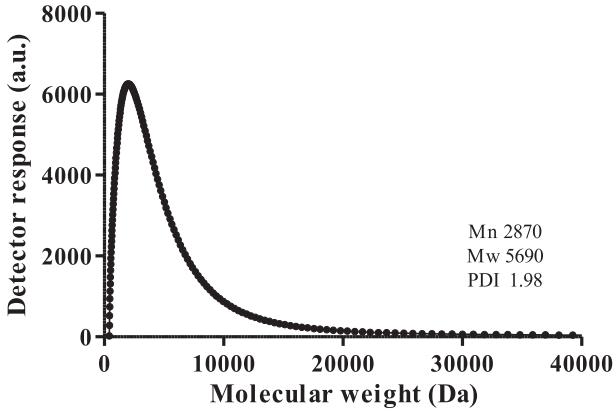

Fig. 1. High performance liquid chromatography (HPSEC) profiles (n = 3) of inulin. *Abbreviations*: arbitrary unit (a.u.); number average molecular weight (Mn); weight average molecular weight (Mw); polydispersity index (PDI).

MyD88, PPARγ and *DEFβ1* gene expressions and lower levels of *AKT1* and *CXCL10* compared to the IPEC-J2 control cells while the blank FS also induced lower levels of *NF-kB1*, *NF-kBIα*, interleukin-8 (*IL8*) and *TNFα* and higher interleukin-1 receptor antagonist (*ILRN1*) mRNA levels in comparison to the control ($P < 0.05$; Fig. 3A and B). In addition to the genes altered by both inulin FS and blank FS, inulin FS also had higher *IL18* and *DEFβ4a* levels and lower *COX2* levels in comparison to the control cells. *AKT1, MAPK14*, *MyD88, DEFβ1, DEFβ4a, NF-kBIα, IL18, IL8, TNFα* and *PPARγ* levels were significantly higher for the inulin FS in comparison to the blank FS.

Inulin DI induced higher adherens (*CDH1*, i.e. e-cadherin) and tight junction gene expression levels (occludin, claudin-3 and zonula occludens-1 (*ZO-1*)) in comparison to the control cells ($P < 0.01$; Fig. 3C) while *CDH1,* mucin 1 (*MUC1*) and claudin-3 mRNA levels of inulin DI were inferior to the ones of both inulin FS and blank FS. *CDH1*, claudin-1, −3, epidermal growth factor receptor (*EGFR*) and *MUC1* expression levels were significantly higher for inulin FS in comparison to inulin DI and the opposite was observed for tricellulin (*MARVELD2*).

Inulin FS and blank FS displayed greater adherens (*CDH1*), tight junction gene expression levels (claudin-1, −3 and *ZO-1*), *EGFR* and

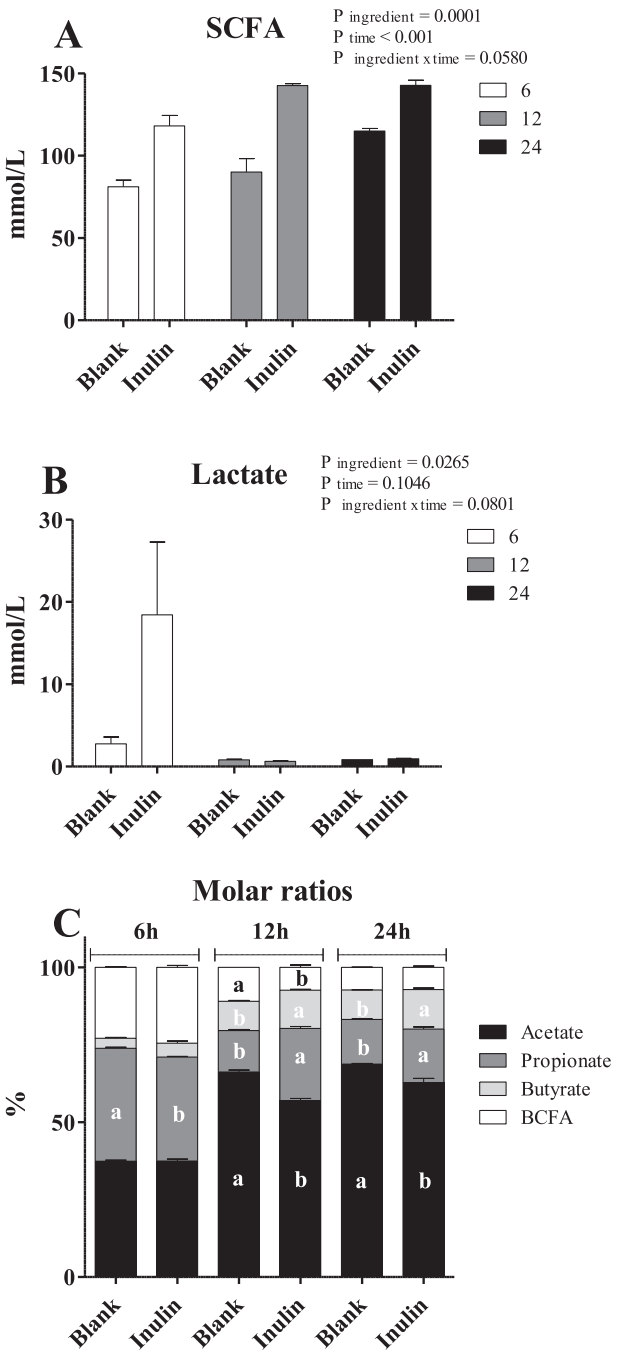

Fig. 2. Fermentation products profile of inulin and blank supernatants (FS) after 6, 12, 24 h of fermentation. (A) SCFA amounts. (B) Lactate amounts. (C). Molar ratios. SCFA = total amount of short-chain fatty acids (acetic + propionic + *i*-butyric + butyric + *i*-valeric + valeric; expressed as mM); acetic, propionic and butyric acid proportions (expressed as % of SCFA). BCFA = branched chain fatty acid proportion (*i*-butyric + *i*-valeric + valeric scaled to SCFA, expressed as %). Values are means of three measurements (n = 3 fermentation vials), with standard error of the mean. For one sampling time, different superscripts denote significant difference ($P < 0.05$). Mean values (n = 3) ± SEM.

MUC1 mRNA levels in comparison to the control cells ($P < 0.05$; Fig. 3C). Inulin FS also had higher caspase 3 (*CASP3*) levels compared to the control cells while blank FS levels showed greater occludin mRNA levels compared to the control cells. *CDH1*, claudin-1, claudin-3 and *EGFR* levels were significantly higher for inulin FS in comparison to blank FS.

4. Discussion

The fermentation capacities of inulin were investigated via the *in vitro* batch fermentation model. Inulin, the high-molecular weight polymer of fructose, mainly composed of soluble fibres, was rapidly and extensively fermented by porcine fecal microbiota which is in line with several studies highlighting the extensive gas capacities of inulin in comparison with other prebiotics using human fecal microbiota as inoculum (Carlson, Erickson, Lloyd, & Slavin, 2018; Fehlbaum et al., 2018). Moreover, we demonstrated in a previous study that the rapidity and extensity of the fermentation were directly correlated to the fructan content of the ingredient (Uerlings et al., 2019a). According to the gas profile, the prebiotic is therefore more likely to be fermented at the end of the small intestine. Moreover, inulin fermentation induced elevated butyrate proportions in comparison to the blank treatment which is in agreement with *in vitro* human models (Grootaert et al., 2009; Van De Wiele, Boon, Possemiers, Jacobs, & Verstraete, 2007). The *in vitro* model using young piglets' feces as inoculum hereby confirmed the ability of young mammals to ferment prebiotics in the early stage of life (Strube, Ravn, Ingerslev, Meyer, & Boye, 2015).

The research aimed to compare the direct (inulin DI) and indirect effects of inulin (inulin FS) on key gut barrier targets and on the immune system by application on IPEC-J2 cells. In addition, a blank FS (feces control) was added to this study, in order to show the added value of the fermentation metabolites of inulin (inulin FS) in comparison to the standard fermentation metabolites present in the fecal inoculum (blank FS).

Decreased *TNFα* and *NF-kBIα* gene expressions and upregulated *CXCL10* and *AKT1* targets were observed with inulin DI, differing from the control cells levels, indicating an activation of the inflammatory response, while other pro-inflammatory targets or signalling pathway proteins remained unaltered. In human intestinal and immune cell cultures (Ortega-González et al., 2014; Zenhom et al., 2011), however, the direct immunomodulatory effect of inulin on cytokine signalling (*PPARγ)* and inflammation pathways (TLR4 and *NF-kB)* has been reported. The concentration of the prebiotic, the origin of the cell line or the time of exposure on the cultured cells might explain such discrepancies.

Application of FS (blank FS and inulin FS) induced conflicting results in terms of immune processes with the upregulation of several pro-inflammatory targets such as *MAPK14*, *NOD1*, *MyD88, PPARγ* and *DEFβ1* and the inhibition of *CXCL10* and *AKT1* targets compared to control cells. In addition, we also observed other immunological reactions such as increased *IL18* and *DEFβ4a* gene expressions following the supplementation with inulin FS which is in contrast with the results of Pham et al. (2018) who did not observe any immune-related gene modification with fermented inulin in HT29-MTX and HT29 cell models. Therefore, so far, it is conceivable that unidentified metabolites dependent of the microbiota present in the fecal inoculum might be responsible for the immune cell-modulating effects of FS as hypothesized by Borowicki et al. (2010) and Stein et al. (2010). In view of the results of the blank FS and the inulin FS, fermented inulin had a mild additional effect on immune-related genes compared with metabolites present in the blank FS as seen by upregulated levels of *AKT1, MAPK14, MyD88, DEFβ1, DEFβ4a, NF-kBIα, IL18, IL8, TNFα* and *PPARγ* with inulin FS.

In the current study, inulin DI upregulated the transcription of tight (claudin-3, occludin, *ZO-1*) and adherens (*CDH1*) junctions in comparison to control cells. Such indicators may predict a beneficial impact of inulin directly on the gut epithelial barrier. Nevertheless, the blank FS and the inulin FS exerted an even greater effect on the barrier function with upregulated tight (claudin-1, -3, *ZO-1*) and adherens junction (*CDH1*) as well as *EGFR* and *MUC1* gene expressions. These results suggest that the fermentation step promotes the production of beneficial metabolites, such as butyrate, which are enhancing intestinal barrier integrity.

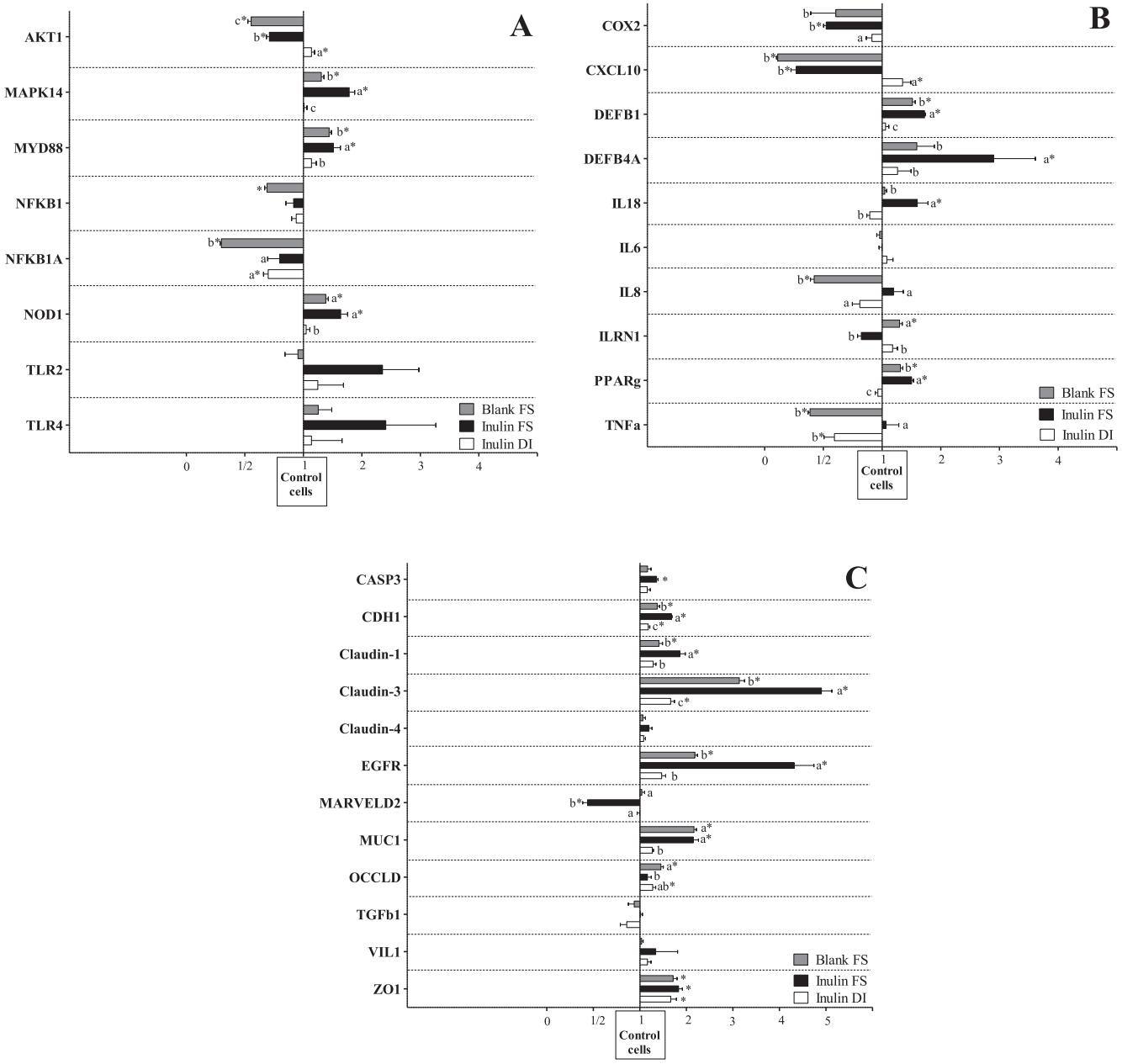

Fig. 3. Impact of inulin DI and FS as well as blank FS collected after 12 h on gene expression in IPEC-J2 cells in comparison to the control cells. (A) Inflammation signalling pathway targets; (B) Pro-inflammatory targets; (C) Barrier integrity targets; Inulin digesta (DI): underwent the *in vitro* hydrolysis and dialysis steps; Inulin fermentation supernatant (inulin FS): subsequently underwent colonic fermentation for 12 h; Blank fermentation supernatant (blank FS): blank FS from the colonic fermentation after 12 h; Values are means of triplicate well-replicates, with standard error of the mean. Geometric mean of RPL13a, GAPDH, PPIA and YWHAZ was used to normalize samples. Figures display the % of difference in comparison to the control cells (sham-treated), considered as 1. Different superscripts letters denote significant difference between blank FS and inulin FS and inulin DI ($P < 0.05$). Superscript symbol (*) denotes significant difference between the treatments and control cells hereby considered as 1 ($P < 0.05$). *Abbreviations*: serine/threonine-protein kinase 1 (*AKT1*); caspase 3 (*CASP3*); E-cadherin (*CDH1*); cyclooxygenase 2 (*COX2*); C-X-C motif chemokine 10 (*CXCL10*); defensin beta (*DEFβ*); epidermal growth factor receptor (*EGFR*); interleukin (*IL*); interleukin-1 receptor antagonist (*ILRN1*); mitogen-activated protein kinase 14 (*MAPK14*); tricellulin (*MARVELD2*); mucin 1 (*MUC1*); myeloid differentiation primary response 88 (*MyD88*); nuclear factor-kappa B (*NF-kB*); nuclear factor-kappa B inhibitor alpha (*NF-kBIα*); nucleotide-binding oligomerization domain-containing protein 1 *(NOD1*); peroxisome proliferator-activated receptor gamma (*PPARγ*); transforming growth factor beta 1 (*TGFβ1*); toll-like receptor (*TLR*); tumor necrosis factor alpha (*TNFα*); villin 1 (*VIL1*); tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*); zonula occludens-1 (*ZO-1*).

Furthermore, our results demonstrate that inulin FS had an additional effect on the expression of gut barrier genes compared to the blank FS, with upregulated *CDH1*, claudin-1, claudin-3 and *EGFR* transcription activities, which in is line with Allsopp et al. (2013) and Pham et al. (2018) who observed an increased transepithelial electrical resistance (TEER) using fermented inulin in human cell lines. One reason for this protective effect might be the production of beneficial SCFAs and in particular butyrate (Fässler et al., 2007; Lux, Scharlau, Schlörmann, Birringer, & Glei, 2012; Schlörmann et al., 2012) which is of physiological significance as a primary source of energy for the colonic epithelial cells (Roediger, 1982). As the maintenance of the gut barrier tightness is an energy-requiring process, it could be postulated that tight and adherens junction strength is promoted via increased cellular energy levels, as hypothetised by Commane et al. (2005), due to butyrate production via the fermentation of inulin. Therefore, we demonstrated the added value of the fermentation metabolites present in

inulin FS compared to the initial metabolites of the blank FS, especially concerning gut barrier integrity. Interestingly, *CASP3*, a protein involved in apoptosis, was upregulated upon treatment with inulin FS in comparison to control cells possibly due to butyrate as this key fermentation product is known to mediate pro-apoptotic effects and is involved in cell death (Munjal, Glei, Pool-Zobel, & Scharlau, 2009). Nevertheless, it is likely that synergistic effects of butyrate with other fermentation metabolites present in the inoculum may increase the health-promoting capacities of the FS and it would be of great interest to highlight potential unidentified metabolites through a metabolomics approach. Nonetheless, with this model, novel prebiotics may be tested for their bioactive properties for future inclusion in young human and pig diets.

In conclusion, a remarkable upregulation of genes related to the intestinal barrier integrity was observed following both blank FS and inulin FS application compared to inulin DI, with the additional effects therefore arising from the fermentation process. Moreover, this response was exacerbated with inulin FS in comparison to the blank FS, mediated by butyrate levels as key bioactive metabolite.

Declaration of Competing Interest

None.

Acknowledgments

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Authors contributions to manuscript

J.U., M.S., J.B., E.W., S.T., G.B. and N.E for Conceptualization, Investigation, Methodology, Supervision and Validation; J.U. for Data curation and Formal analysis; E.W., S.T., G.B. and N.E for Resources and Funding acquisition; J.U. for Visualisation, Roles/Writing; original draft; J.U., M.S., J.B., E.W., S.T., G.B. and N.E Writing – review and editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.103855.

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