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Evaluation of the *in vitro* effects of the increasing inclusion levels of yeast β-glucan, a casein hydrolysate and its 5 kDa retentate on selected bacterial populations and strains commonly found in the gastrointestinal tract of pigs†

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Previously, the 5 kDa retentate (5kDaR) of a casein hydrolysate (CH) and yeast β-glucan (YBG) were identified as promising anti-inflammatory dietary supplements for supporting intestinal health in pigs post-weaning. However, their direct effects on intestinal bacterial populations are less well-known. The main objectives of this study were to determine if the increasing concentrations of the CH, 5kDaR and YBG individually, can: (1) alter the bacterial and short-chain fatty acid profiles in a weaned pig faecal batch fermentation assay, and (2) directly influence the growth of selected beneficial (*Lactobacillus plantarum, L. reuteri, Bifidobacterium thermophilum)* and pathogenic (Enterotoxigenic *Escherichia coli*, *Salmonella* Typhimurium) bacterial strains in individual pure culture growth assays. The potential of CH as a comparable 5kDaR substitute was also evaluated. The 5kDaR increased lactobacilli counts and butyrate concentration in the batch fermentation assay ($P < 0.05$) and increased *L. plantarum* (linear, $P < 0.05$), *L. reuteri* (quadratic, $P < 0.05$) and *B. thermophilum* (linear, $P < 0.05$) counts and reduced *S. typhimurium* (quadratic, $P = 0.058$) counts in the pure culture growth assays. CH increased butyrate concentration ($P < 0.05$) in the batch fermentation assay. YBG reduced *Prevotella* spp. counts ($P < 0.05$) and butyrate concentration ($P < 0.05$) in the batch fermentation assay. Both CH and YBG had no major effects in the pure culture growth assays. In conclusion, the 5kDaR had the most beneficial effects associated with increased counts of *Lactobacillus* and *Bifidobacterium* genera and butyrate production and reduced *S. typhimurium* counts *in vitro* indicating its potential to promote gastrointestinal health.

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Introduction

Gastrointestinal functionality, homeostasis and health are greatly influenced by the residing microbiota.[1] Two widely known beneficial constituents of this microbial community are the genera *Lactobacillus* and *Bifidobacterium* due to their contributions to carbohydrate metabolism, colonisation resistance against pathogens, immunomodulation and maintenance of intestinal barrier function.[2–5] *Prevotella* genus is another important commensal of the gastrointestinal microbiota involved in the degradation of complex dietary carbohydrates providing the host with energy and beneficial metabolites such as short-chain fatty acids (SCFA).[6–8] From a health perspective, the *Enterobacteriaceae* family is a significant intestinal population, as it includes many human and swine pathogens such as *Escherichia coli* and *Salmonella enterica subsp. enterica* serovars (*e.g.* Typhimurium, Enteritidis).[9–12] Disturbances to the composition and/or metabolic activities of the gastrointestinal microbiota can result in dysbiosis.[13] Post-weaning diarrhoea in pigs is a classic example of a dysbiotic condition whereby pigs have reduced feed intake, resulting in reduced growth and performance and leading to significant economic losses.[14] In humans, dysbiosis is associated with a wide range of diseases and disorders such as inflammatory bowel disease, colorectal cancer and metabolic disorders.[13]

Diet is an important driver of the composition of the gastrointestinal microbiota. Extensive research has been carried out to identify dietary supplements which promote the growth of

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beneficial bacteria and/or inhibit the colonisation of pathogens. Of interest are the bioactive peptides derived from the bovine milk proteins, casein and whey. These peptides exhibit a broad range of biological activities such as antihypertensive,[15] antioxidative,[16] immunomodulatory[17–19] and antibacterial against various pathogenic and spoilage bacteria.[20–24] Another interesting finding is the growth-promoting and protective properties of milk bioactive peptides to beneficial bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp.[19,23,25,26] Furthermore, there is evidence that these bioactive peptides can be multifunctional. For instance, a casein peptide exhibiting antibacterial, antioxidant and antihypertensive capacity has been previously identified.[22]

Recently, dietary supplementation of weaned pigs with a 5 kDa retentate (5kDaR) of a sodium caseinate hydrolysate (CH) combined with yeast β-glucan (YBG), a non-digestible polysaccharide with known immunomodulatory activity,[27,28] alleviated the negative impact of weaning and improved faecal consistency.[29] It was hypothesised that the YBG protected the 5kDaR from hydrolysis in the small intestine. While the 5kDaR has established anti-inflammatory activity both *in vitro*[30,31] and in the weaned pig model,[29] its direct effects on the GIT microbiota, including, antimicrobial activity against pathogens and/or stimulatory effects on beneficial bacterial species are still undetermined. To further understand the mode of action of the 5kDaR, a batch fermentation assay using faecal inoculum and pure culture growth assays were employed as these are established methods with which to investigate the effects of bioactive compounds on selected bacterial groups within a microbial community and on specific bacterial strains.[32,33] The primary objective of this study was to investigate the effects of the increasing concentrations of CH, its 5kDaR and YBG individually on selected bacterial populations and on SCFA production in the faeces of weaned pigs using a batch fermentation assay. Based on the results of the primary objective, we then determined if the beneficial changes were evident in specific bacterial species. Thus, the second objective of this study was to determine whether the increasing concentrations of CH, its 5kDaR and YBG individually enhance the growth of the beneficial genera *Lactobacillus* spp. and *Bifidobacterium* spp. and/or have antimicrobial activity specific to animal and foodborne pathogens in pure culture growth assays. As the production of the 5kDaR is expensive, the final objective of this study was to evaluate the potential of CH as a comparable substitute to 5kDaR.

Materials and methods

An outline of the experimental processes of the study is provided in Fig. S1.†

Casein hydrolysates and YBG

The CH and its 5kDaR were produced from the hydrolysis of sodium caseinate (NaCas, ≈90% w/w protein, Kerry Food Ingredients, Listowel, Ireland) derived from bovine milk and have been described previously.[31] The YBG was derived from *Saccharomyces cerevisiae* (Biothera Pharmaceuticals, Inc., Eagan, MI, USA) and has been described previously.[27] All compounds were stored at room temperature in sealed containers.

Batch fermentation assay

Faecal inoculum. Faeces from 29 newly weaned crossbred pigs (Large White × Landrace) from a commercial farm (Perma pigs Limited, Co. Kildare, Ireland) were collected and pooled. The pooled faeces were aliquoted and stored at −20 °C. The faecal inoculum (FI) was prepared one day prior to the batch fermentation assay by performing a 5-fold dilution of the pooled faeces on weight basis (1 : 5 w/v) in pre-reduced phosphate buffered saline (Sigma-Aldrich, St Louis, MO, USA) after the addition of oxyrase (Sigma-Aldrich, St Louis, MO, USA), an enzyme that removes oxygen from the broth. The FI was stored at 4 °C within a sealed container with anaerobic conditions established using AnaeroGen 2.5 L sachets (Thermo Fisher Scientific, Waltham, MA, USA).[34]

Fermentation assay. The modified batch fermentation assay was designed based on previous studies.[34,35] The media and reagents used were purchased by Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated. The composition of the fermentation medium (FM-medium) was as follows: 5 g L^{-1} yeast extract (Y1625), 10 g L^{-1} ascorbic acid (A4544), 10 g L^{-1} sodium acetate (S5636), 5 g L^{-1} $(NH_4)_2SO_4$ (A3920), 2 g L^{-1} urea (U5378), 0.2 g L^{-1} $MgSO_4 7H_2O$ (M2773), 0.01 g L^{-1} $FeSO_4 7H_2O$ (F8633), 0.007 g L^{-1} $MnSO_4 xH_2O$ (M7899), 0.01 g L^{-1} NaCl (S5886), 1 ml L^{-1} Tween 80 (P4780), 0.05 g L^{-1} hemin (51280) and 0.5 g L^{-1} L-cysteine hydrochloride (C1276). The pH was adjusted to 7.0. The FM-medium was autoclaved for dissolution and sterilisation and stored at 4 °C. The tested casein hydrolysates and YBG were diluted in a final volume of 21 ml FI/FM medium at 1, 2.5 and 5 mg ml^{-1} concentrations. The FI : FM-medium ratio was 1 : 10 v/v. The batch fermentation was carried out in glass tubes (PYREX™ Disposable Round-Bottom Rimless Glass Tubes, Fisher Scientific, Co. Dublin, Ireland) with rubber stoppers (Saint-Gobain Rubber stopper grey blue 17/22 x H 25MM, VWR, Co. Dublin, Ireland). Anaerobic conditions were established and maintained by the addition of oxyrase and CO_2 flushing. Control tubes (0 mg ml^{-1}) containing only FI and FM-medium were also included. All tubes were incubated at 39 °C for 24 h with gentle stirring (100 rpm). A volume of 5 ml fermentation broth was collected at 0, 10 and 24 h in duplicate tubes. The collected samples were centrifuged at 12 000*g* for 5 minutes. The supernatants and pellets were stored in −20 °C until further analysis. All experiments were repeated on three independent occasions, hence $n = 3$ biological replicates.

Short chain fatty acid (SCFA) analysis

The SCFA profile of the supernatants was determined using gas liquid chromatography as described previously.[36] Each supernatant sample (1 g) was diluted with distilled water (2.5 × weight of sample) and centrifuged at 1400*g* for 10 minutes (Sorvall GLC-2 B laboratory centrifuge, DuPont, Wilmington,

DE, USA). One ml of the subsequent supernatant and 1 ml of internal standard (0.05% 3-methyl-*n*-valeric acid in 0.15 M oxalic acid dihydrate) were mixed with 3 ml distilled water. The reaction mixture was centrifuged at 500*g* for 10 min and the supernatant was filtered through 0.45 PTFE (polytetrafluoroethylene) syringe filter into a chromatographic sample vial. An injection volume of 1 μl was injected into a Varian 3800 GC equipped with an EC™ 1000 Grace column (15 m × 0.53 mm I.D.) with 1.20 μm film thickness. The temperature programme set was 75–95 °C increasing by 20 °C per minute, which was held for 30 seconds. The detector and injector temperature were 280 and 240 °C, respectively, while the total analysis time was 12.42 minutes.

Quantification of bacterial groups using quantitative real time polymerase chain reaction (QPCR)

DNA extraction. Microbial genomic DNA was extracted using QIAamp Fast DNA stool mini kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions. The DNA quantity and quality were evaluated using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Bacterial primers. The list of the domain-, family- or genus-specific primers targeting the 16S rRNA gene of the selected bacterial groups are provided in Table 1.

Plasmid and standard curve preparation. Bacterial genomic DNA from *Bifidobacterium thermophilum* (DSMZ 20210), *Lactobacillus plantarum (*DSMZ 20174) and *S. typhimurium* phage type (PT) 12 was extracted from pure cultures using DNeasy® Blood & Tissue kit (Qiagen, West Sussex, UK), whereas for *Prevotella* spp. bacterial genomic DNA of *P. bryantii* (DSMZ 11371) was used. Bacterial strains and genomic DNA were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) except for *S. typhimurium* PT 12.[37] The targeted genes were amplified with a conventional PCR with the respective primers and genomic locations being outlined in Table S1.† The amplicons were incorporated into a vector using the TOPO™ TA Cloning™ Kit for Sequencing (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). One Shot™ TOP10 Chemically Competent *E. coli* were transformed according to the manufacturer's instructions using a heat shock method. LB agar (Lennox, Co. Dublin, Ireland) plates containing ampicillin (100 μg ml^{-1}) were inoculated with the bacterial culture (50 μl) using a sterile plate spreader and incubated at 37 °C overnight. Individual colonies were re-plated, screened for the presence of the plasmid and preserved on cryoprotective beads (TS/71-MX, Protect Multipurpose, Technical Service Consultants Ltd, Lancashire, UK) that were stored at −80 °C. The transformed *E. coli* was re-cultured by transferring a single cryoprotective bead in 200 ml LB Broth Base (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) containing ampicillin and incubated at 37 °C for 18 h at 150 rpm. The plasmids carrying the target genes were purified on a large scale using the GenElute™ HP Plasmid Maxiprep kit (Sigma-Aldrich, St Louis, MO, USA) and linearised using APA1 (Promega, Madison, WI, USA) restriction enzyme according to the manufacturer's instructions. The linearised plasmids were further purified using the GenElute™ PCR Clean-Up kit (Sigma-Aldrich, St. Louis, MO, USA). The purified, linearized plasmids were quantified spectrophotometrically and copy number per μl was determined using an online tool which employs the formula mol g^{-1} × molecules per mol = molecules per g using Avogadro's number, 6.022 × 10^{23} molecules per mol provided by the URI Genomics & Sequencing Center (http://cels.uri.edu/gsc/cndna.html). Standard curves were prepared using 5-fold serial dilutions for the purposes of QPCR.

QPCR. The final reaction volume (20 μl) included 3 μl template DNA, 1 μl of each primer (10 μM), 5 μl nuclease-free water and 10 μl of Fast SYBR® Green Master Mix (Applied Biosystems. Foster City, CA, USA) for the *Lactobacillus* spp. or GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) for the remaining bacterial groups. All QPCR reactions were performed in duplicate and were carried out on ABI 7500 Fast PCR System (Applied Biosystems, Foster City, CA, USA). The cycling conditions included a denaturation step of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Dissociation curves were generated to confirm the specificity of the resulting PCR products. In addition, the PCR products were visualised on an agarose gel stained with ethidium bromide, to ensure that the PCR products from the sample and plasmid were of equal size. The efficiency of each QPCR reaction was established by plotting the threshold cycles (Ct) derived from 5-fold serial dilutions of the plasmid against their arbitrary quantities and only assays exhibiting 90–110% efficiency and single products were used in this study. Bacterial counts were determined using a standard curve derived from the mean Ct value and the log transformed gene copy number of the plasmid and expressed as log transformed gene copy number per gram of digesta (LogGCN per g digesta).

Table 1 List of forward and reverse primers used for the bacterial quantification by QPCR

Target bacterial group	Forward primer (5′-3′), Reverse primer (5′-3′)	Amplicon length (bp)	T_m (°C)
Total bacteria	F: GTGCCAGCMGCCGCGGTAA, R: GACTACCAGGGTATCTAAT	291	64.2, 52.4
Lactobacillus spp.	F: AGCAGTAGGGAATCTTCCA, R: CACCGCTACACATGGAG	341	54.5, 55.2
Bifidobacterium spp.	F: GCGTGCTTAACACATGCAAGTC, R: CACCCGTTTCCAGGAGCTATT	125	60.3, 59.8
Enterobacteriaceae	F: ATGTTACAACCAAAGCGTACA, R: TTACCYTGACGCTTAACTGC	185	54.0, 56.3
Prevotella spp.	F: CACRGTAAACGATGGATGCC, R: GGTCGGGTTGCAGACC	514	58.3, 56.9

bp, base pairs; T_m, melting temperature.

Bacterial strains and growth conditions

The strains *L. plantarum* (DSMZ 20174), *L. reuteri* (DSMZ 20016) and *B. thermophilum* (DSMZ 20210) were selected as they are commensals with well characterised health-promoting properties. The selected pathogenic strains were the enterotoxigenic *E. coli* (ETEC) O149A+, a predominant cause of post-weaning diarrhoea in weaned piglets, and *S. typhimurium* PT12, an important foodborne pathogen. Specific information for each bacterial strain is provided in Table S2.† *L. plantarum*, *B. thermophilum* and *S. typhimurium* strains were as above, *L. reuteri* was purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures and ETEC was kindly provided from the Central Veterinary Research laboratory (DAFM-Laboratories Backweston, Co. Kildare, Ireland).

All bacterial strains were revived from cryoprotective beads that were stored at −20 °C. *L. plantarum* and *L. reuteri* were inoculated in 10 ml volume of de Man, Rogosa and Sharpe broth (MRS, Oxoid Ltd, Hampshire, UK) and incubated aerobically at 37 °C for 48 h. *B. thermophilum* was streaked onto MRS agar (MRSA, Oxoid Ltd, Hampshire, UK) and incubated anaerobically at 37 °C for 48 h. Anaerobic conditions were established within sealed containers using AnaeroGen 2.5 L and 3.5 L sachets (Thermo Fisher Scientific, Waltham, MA, USA). ETEC and *S. typhimurium* were inoculated in 10 ml Tryptone Soya Broth (TSB, Oxoid Ltd, Hampshire, UK) and incubated aerobically at 37 °C for 48 h. All bacterial strains were sub-cultured by transferring 1 ml of the previous culture to 9 ml of the respective sterile medium and incubated aerobically at 37 °C for 24 h. In the case of *B. thermophilum* a few colonies were inoculated into 10 ml MRS and incubated anaerobically at 37 °C for 24 h. Lactobacilli and ETEC and *S. typhimurium* cultures were additionally streaked on MRSA and Tryptone Soya Agar (TSA, Oxoid Ltd, Hampshire, UK) respectively to ensure purity. The 24 h cultures were used for the subsequent assays.

Pure culture growth assays

The assays were designed based on methodologies from previous experiments with some modifications.[38–40] The assays were carried out in 96-well microtiter plate (CELLSTAR, Greiner Bio-One, Kremsmünster, Austria). Casein hydrolysates and YBG were diluted in 10% MRS and 10% TSB at a working concentration of 4 mg ml^{-1} and stored at 4 °C. Contamination checks were performed regularly to ensure the sterility of the subsequent assays. Further 2-fold dilutions (2–0.25 mg ml^{-1}) were performed each time prior to the assay. All bacterial strains were diluted in 10% medium (MRS or TSB) to obtain an inoculum of 10^6–10^7 CFU (colony-forming unit) per ml with initial bacterial enumeration being performed each time. 100 μl of each compound and each dilution was transferred to duplicate wells and 100 μl inoculum was added. Control wells containing 100 μl of 10% medium and 100 μl inoculum were also included. To evaluate the sterility, blank wells containing 100 μl of 10% medium and 100 μl of each dilution of each compound were considered. Plates were agitated gently to ensure thorough mixing and incubated aerobically at 37 °C for 18 h, apart from *B. thermophilum* that was incubated anaerobically.

After incubation, spread plating was used to determine both the bacterial viability and counts at the increasing concentrations of each compound. A modified Miles and Misra method was used to determine the final bacterial concentration.[41] Briefly, the content of the wells was mixed by pipetting and 25 μl from each well was transferred to 225 μl Maximum Recovery Diluent (MRD, Oxoid Ltd, Hampshire, UK). Following a 10-fold serial dilution (10^{-1}–10^{-8}), 20 μl was transferred onto MRSA for *L. plantarum*, *L. reuteri* and *B. thermophilum* and TSA for ETEC and *S. typhimurium*. Plates were incubated aerobically at 37 °C for 24 h with the exception of *B. thermophilum* which was incubated anaerobically at 37 °C for 48 h. The dilution resulting in 5–50 colonies was selected to calculate CFU per ml using the formula CFU per ml = Average colony number × 50 × dilution factor. Bacterial counts were logarithmically transformed (logCFU per ml) for the subsequent statistical analysis. All experiments were carried out with technical replicates on three independent occasions, hence, $n = 3$ biological replicates.

Statistical analysis

Statistical analysis was performed using Statistical Analysis Software (SAS) 9.4 (SAS Institute, Cary, NC, USA). All data were initially tested for normality using PROC UNIVARIATE procedure.

Batch fermentation assay. To determine the robustness of the assay over time, the bacterial counts of selected bacterial groups in the untreated control flasks ($n = 9$) were analysed by repeated measures analysis using the PROC MIXED procedure[42] with the fixed effect of time (0, 10 and 24 h). The bacterial counts of selected bacterial groups and SCFA concentrations at 10 and 24 h were analysed using PROC GLM procedure. The model assessed the effect of the compound concentration with the experimental unit being the biological replicate. 0 h was used as a covariate.

Pure culture growth assay. The bacterial counts from the pure culture growth assays were analysed using PROC GLM procedure for the presence of linear and quadratic effects of concentration for each compound. The biological replicate was the experimental unit. The LSMEANS statement was additionally used to calculate the least-square means and the standard error of the means (SEM).

Probability values of $P < 0.05$ denote statistical significance, whereas probability values between 0.05 and 0.1 are considered numerical tendencies. Results are presented as least-square means ± SEM.

Results

Evaluation of the robustness of the batch fermentation assay

The ability of the batch fermentation assay to maintain a complex faecal bacterial community was evaluated by determining the changes in the counts of the selected bacterial groups in

Table 2 Effect of time on the log transformed gene copy number per g faeces of the different bacterial groups (Least-square means with their standard errors)

Bacterial group (logGCN per g faeces)	Time			SEM	*P*-Value
	0 h	10 h	24 h		Time effect
Total bacteria	9.24[b]	8.99[a]	9.70[c]	0.085	<0.001
Lactobacilli	8.20[a]	8.42[b]	8.55[c]	0.060	<0.001
Bifidobacteria	6.11[a]	6.21[b]	6.53[c]	0.053	<0.001
Enterobacteriaceae	6.93[a]	8.01[b]	8.11[b]	0.048	<0.001
Prevotella spp.	8.82[c]	7.87[b]	7.75[a]	0.057	<0.001

logGCN per g faeces, log transformed gene copy number per gram of faeces. [a,b,c]Mean values within a row with different superscript letter were significantly different ($P < 0.05$).

the untreated control tubes (0 mg ml^{-1}) at the different time points, presented in Table 2. The counts of total bacteria initially decreased at 10 h before increasing at 24 h ($P < 0.05$). The counts of lactobacilli increased to the greatest extent at 10 h ($P < 0.05$). The counts of bifidobacteria increased predominantly at 24 h ($P < 0.05$). The counts of *Enterobacteriaceae* increased at 10 h solely ($P < 0.05$). The counts of *Prevotella* spp. decreased predominantly at 10 h ($P < 0.05$).

Effects of casein hydrolysates and YBG on the selected faecal bacterial populations

The effects of the different concentrations of CH, 5kDaR and YBG on the selected bacterial populations in the batch fermentation assay are presented in Table 3.

CH. CH had no effect on the counts of total bacteria, lactobacilli, bifidobacteria, *Enterobacteriaceae* and *Prevotella* spp. at all tested concentrations and time points ($P > 0.05$).

5kDaR. There was no effect of any 5kDaR concentration on total bacterial counts at 10 h ($P > 0.05$), however, these were increased at all 5kDaR concentrations at 24 h compared to the control ($P < 0.05$). There was an increase in lactobacilli counts at 5 mg ml^{-1} at 10 h compared to the control ($P < 0.05$), however, no effects were observed at all tested concentrations at 24 h ($P > 0.05$). 5kDaR had no effect on the counts of bifidobacteria, *Enterobacteriaceae* and *Prevotella* spp. at all tested concentrations and time points ($P > 0.05$).

YBG. There was a decrease in *Prevotella* spp. counts at 2.5 and 5 mg ml^{-1} at both time points compared to the control ($P < 0.05$). YBG had no effect on the counts of total bacteria, lactobacilli, bifidobacteria and *Enterobacteriaceae* at all tested concentrations and time points ($P > 0.05$).

Table 3 Effects of CH, 5kDaR and YBG on the log transformed gene copy number per g faeces of selected bacterial groups at 10 and 24 h in the batch fermentation assay (Least-square means with their standard errors)

Compound	Sampling point	Bacterial group (logGCN per g faeces)	Compound concentration (mg ml^{-1})				SEM	*P*-Value
			0	1	2.5	5		
CH	10 h	Total bacteria	8.93	9.10	9.16	9.06	0.158	0.772
		Lactobacilli	8.39	8.53	8.70	8.69	0.074	0.061
		Bifidobacteria	6.18	6.16	6.24	6.12	0.053	0.528
		Enterobacteriaceae	8.00	8.07	8.19	8.11	0.061	0.222
		Prevotella spp.	7.99	8.08	8.17	8.05	0.047	0.134
	24 h	Total bacteria	9.83	9.85	9.94	9.95	0.047	0.242
		Lactobacilli	8.65	8.60	8.73	8.67	0.051	0.399
		Bifidobacteria	6.65	6.65	6.61	6.55	0.034	0.211
		Enterobacteriaceae	8.16	8.09	8.21	8.17	0.046	0.427
		Prevotella spp.	7.96	7.84	7.94	7.87	0.049	0.355
5kDaR	10 h	Total bacteria	8.73	8.77	8.49	9.05	0.151	0.169
		Lactobacilli	8.44[a]	8.52[a]	8.47[a]	8.75[b]	0.058	0.028
		Bifidobacteria	6.32	6.41	6.43	6.43	0.033	0.113
		Enterobacteriaceae	7.92	7.99	8.01	8.12	0.071	0.333
		Prevotella spp.	7.81	7.87	7.85	7.97	0.045	0.150
	24 h	Total bacteria	9.54[a]	9.76[b]	9.83[b]	9.88[b]	0.061	0.022
		Lactobacilli	8.58	8.64	8.61	8.72	0.039	0.148
		Bifidobacteria	6.60	6.70	6.67	6.68	0.032	0.231
		Enterobacteriaceae	7.96	7.94	8.02	8.05	0.048	0.424
		Prevotella spp.	7.68	7.73	7.73	7.75	0.054	0.810
YBG	10 h	Total bacteria	9.34	8.80	8.91	9.05	0.121	0.072
		Lactobacilli	8.42	8.33	8.24	8.31	0.087	0.561
		Bifidobacteria	6.14	6.01	5.90	5.79	0.090	0.118
		Enterobacteriaceae	8.14	7.97	8.04	8.06	0.044	0.143
		Prevotella spp.	7.82[c]	7.66[bc]	7.58[ab]	7.42[a]	0.053	0.006
	24 h	Total bacteria	9.74	9.69	9.75	9.56	0.129	0.737
		Lactobacilli	8.44	8.45	8.36	8.36	0.086	0.802
		Bifidobacteria	6.36	6.27	6.19	6.04	0.081	0.116
		Enterobacteriaceae	8.23	7.87	7.98	8.07	0.079	0.072
		Prevotella spp.	7.61[b]	7.41[ab]	7.38[a]	7.22[a]	0.071	0.038

logGCN per g faeces, log transformed gene copy number per gram of faeces. [a,b,c]Mean values within a row with different superscript letter were significantly different ($P < 0.05$).

Effects of casein hydrolysates and YBG on SCFA production

The effects of the different concentrations of CH, 5kDaR and YBG on the SCFA production in the batch fermentation assay are presented in Table 4.

CH. There was no effect of any CH concentration at 10 h ($P > 0.05$), however, butyrate concentration was increased at all CH concentrations at 24 h compared to the control ($P < 0.05$). CH had no effect on total SCFA, acetate and propionate production at all tested concentrations and time points ($P > 0.05$).

5kDaR. There was no effect of any 5kDaR concentration at 10 h ($P > 0.05$), however, butyrate concentration was increased at 1 and 2.5 mg ml^{-1} at 24 h compared to the control ($P < 0.05$). 5kDaR had no effect on total SCFA, acetate and propionate production at all tested concentrations and time points ($P > 0.05$).

YBG. There was a decrease in butyrate concentration at 1 and 2.5 mg ml^{-1} at 10 h compared to the control ($P < 0.05$). Butyrate concentration was numerically reduced at 5 mg ml^{-1} at 10 h compared to the control ($P = 0.074$). However, no effects on butyrate production were observed at all tested concentrations at 24 h ($P > 0.05$). YBG had no effect on total SCFA, propionate and acetate production at all tested concentrations and time points ($P > 0.05$).

Stimulatory and antibacterial properties of casein hydrolysates and YBG in pure bacterial cultures

The effects of the increasing concentrations of CH, 5kDaR and YBG on the growth of a panel of selected beneficial (*L. plantarum*, *L. reuteri*, *B. thermophilum*) and pathogenic (ETEC, *S. typhimurium*) bacterial strains were evaluated in the pure culture growth assays and are presented in Table 5.

CH. There was a linear increase in the counts of *L. plantarum* and *S. typhimurium* in response to the increasing CH concentrations ($P < 0.05$). CH did not have any effect on the counts of *L. reuteri, B. thermophilum* and ETEC at all concentrations tested ($P > 0.05$).

5kDaR. There was a beneficial linear increase in the counts of *L. plantarum* and *B. thermophilum i*n response to the increasing 5kDaR concentrations ($P < 0.05$). There was a beneficial quadratic effect of the increasing 5kDaR concentrations observed in *L. reuteri* with maximum counts observed at 0.25 mg ml^{-1} ($P < 0.05$). There was a beneficial quadratic effect of the increasing 5kDaR concentrations observed in *S. typhimurium*, with lowest counts observed at 1 mg ml^{-1} ($P < 0.05$). 5kDaR had no effect on the ETEC counts at all concentrations tested ($P > 0.05$).

YBG. There was a quadratic effect of the increasing YBG concentration in *L. plantarum*, *L. reuteri* and ETEC counts ($P < 0.05$). The inclusion of 1 mg ml^{-1} was identified as the optimal concentration to stimulate the growth of *L. plantarum* and *L. reuteri* counts, while decreasing ETEC counts. YBG had no effect on the counts of *B. thermophilum* or *S. typhimurium* at all concentrations tested ($P > 0.05$).

Discussion

Gastrointestinal dysbiosis is associated with compromised health in both animals and humans. Dietary supplementation

Table 4 Effects of CH, 5kDaR and YBG on the SCFA profile at 10 and 24 h in the batch fermentation assay (Least-square means with their standard errors)

			Compound concentration (mg ml^{-1})					
Compound	Sampling point	SCFA (mmol per lit)	0	1	2.5	5	SEM	*P*-Value
CH	10 h	Acetate	27.71	26.93	36.18	21.23	9.602	0.785
		Butyrate	0.79	0.37	1.01	0.90	0.423	0.083
		Propionate	0.35	0.51	1.22	0.40	0.761	0.924
		Total SCFA	28.14	28.75	40.82	21.97	11.372	0.768
	24 h	Acetate	25.35	24.85	28.46	27.63	1.581	0.436
		Butyrate	0.33^{a}	2.20^{b}	1.89^{b}	1.63^{b}	0.227	0.032
		Propionate	0.38	0.47	0.55	0.49	0.154	0.893
		Total SCFA	26.58	28.46	31.75	30.15	1.599	0.302
5kDaR	10 h	Acetate	24.17	22.14	22.80	21.40	2.915	0.918
		Butyrate	0.49	0.34	0.35	0.41	0.072	0.489
		Propionate	0.30	0.29	0.28	0.22	0.054	0.739
		Total SCFA	25.32	23.13	23.79	22.38	3.106	0.919
	24 h	Acetate	24.57	24.39	26.86	21.74	4.303	0.886
		Butyrate	0.37^{a}	1.41^{b}	1.46^{b}	0.82^{a}	0.160	0.006
		Propionate	0.34	0.42	0.46	0.35	0.054	0.365
		Total SCFA	25.68	27.07	29.15	23.14	4.399	0.827
YBG	10 h	Acetate	27.49	24.09	22.89	24.15	1.488	0.242
		Butyrate	0.71^{b}	0.40^{a}	0.41^{a}	0.51^{ab}	0.068	0.046
		Propionate	0.73	0.49	0.49	0.57	0.211	0.823
		Total SCFA	29.75	25.41	24.29	25.82	1.682	0.203
	24 h	Acetate	26.39	25.07	23.35	24.44	0.889	0.197
		Butyrate	1.38	1.38	1.33	1.19	0.056	0.147
		Propionate	0.66	0.57	0.51	0.54	0.032	0.061
		Total SCFA	28.88	27.44	25.53	26.54	0.988	0.192

SCFA, short-chain fatty acids. ^{a,b}Mean values within a row with different superscript letter were significantly different ($P < 0.05$).

Table 5 Bacterial counts following exposure to increasing concentrations of CH, 5kDaR and YBG in the pure culture growth assays (Least-square means with their standard errors)

		Final bacterial concentration (logCFU per ml)						*P*-Value	
Compound	Bacterial strain	0 mg ml^{-1}	0.25 mg ml^{-1}	0.5 mg ml^{-1}	1 mg ml^{-1}	2 mg ml^{-1}	SEM	Linear effect	Quadratic effect
CH	*L. plantarum*	7.88	8.04	7.96	8.09	8.21	0.066	0.001	0.500
	L. reuteri	7.72	7.64	7.70	7.49	7.67	0.079	0.417	0.088
	B. thermophilum	6.43	6.57	6.58	6.60	6.79	0.204	0.127	0.852
	ETEC	8.55	8.67	8.61	8.42	8.72	0.085	0.440	0.166
	S. typhimurium	8.92	8.86	9.08	9.03	9.15	0.050	0.002	0.533
5kDaR	*L. plantarum*	7.67	7.91	7.86	8.19	8.45	0.079	<0.001	0.896
	L. reuteri	7.02	7.62	7.70	7.61	7.70	0.108	0.011	0.049
	B. thermophilum	7.00	7.27	7.31	7.41	7.88	0.127	<0.001	0.877
	ETEC	8.78	8.65	8.65	8.68	8.71	0.077	0.984	0.354
	S. typhimurium	9.30	8.80	8.77	8.68	8.73	0.194	0.017	0.058
YBG	*L. plantarum*	7.18	7.29	7.46	7.58	7.50	0.107	0.008	0.041
	L. reuteri	7.08	7.48	7.60	7.58	7.37	0.204	0.027	0.041
	B. thermophilum	6.44	6.73	6.60	6.38	6.26	0.387	0.330	0.877
	ETEC	8.67	8.46	8.40	8.37	8.58	0.060	<0.001	0.001
	S. typhimurium	8.87	9.00	8.97	8.91	9.00	0.075	0.863	0.629

CFU, colony-forming unit.

with natural bioactives may contribute to the maintenance of a healthy gastrointestinal microbiota by stimulating beneficial bacterial populations such as lactobacilli and bifidobacteria or by inhibiting pathogen overgrowth. Previous research identified 5kDaR and YBG as potential natural dietary supplements for weaned pigs.[29] Hence, the main objective of this study was to identify the direct effects of the 5kDaR and YBG on key bacterial populations and species in the gut. As the production of 5kDaR from the parent CH is expensive, the potential of CH to be used as a comparable substitute to 5kDaR was also investigated in this study.

The fermentation conditions used in this study supported the growth of the targeted bacterial groups and maintained a relatively complex microbial community. Batch fermentation is a well-recognised screening assay for the *in vitro* evaluation of the direct effects of the tested compounds on the faecal microbiota.[33,43] The reductions in bacterial counts of total bacteria and *Prevotella* spp. between 0 and 10 h are most likely due to the loss of viable bacteria caused by freezing and thawing of the faeces. QPCR does not differentiate between live and dead cells resulting in higher bacterial counts at 0 h.[44] Consequently, the 0 h time point represents a screenshot of the pooled faecal microbiota of the sampled animals and an overestimation of the viable counts of each bacterial group in the batch fermentation assay, whereas the 10 h time point represents the surviving faecal microbiota adapted to the new environment and treatments. Finally, the growth-stimulating effects of the different tested compounds most likely resulted in a more rapid depletion of nutrients and accumulation of toxic metabolites, common characteristics of the closed and uncontrolled environment of the batch fermentation assay,[43] which probably explain the lack of any further effect in some instances (*e.g.* 5kDaR increased lactobacilli at 10 h but not at 24 h.

The total bacteria as well as the lactobacilli and bifidobacteria that contribute significantly to the gastrointestinal health and growth of the host[2,4,5,45,46] were among the bacterial groups measured in the batch fermentation assay. The *Enterobacteriaceae* family was also included in this study as it is considered a reliable marker of a dysbiotic microbiota and intestinal dysfunction that contribute to the development of gastrointestinal and metabolic disorders and diseases in humans and pigs.[14,47,48] Of the three compounds tested, 5kDaR increased the counts of total bacteria and lactobacilli with no effect on bifidobacteria and *Enterobacteriaceae* counts, while CH and YBG had no major effects on these bacterial populations. The ability of 5kDaR to stimulate lactobacilli growth within a complex microbial community was in agreement with previous studies in which the ileal and colonic counts of this bacterial population were increased in weaned and growing pigs supplemented with casein-derived peptides.[49,50]

An additional bacterial group that was measured in the batch fermentation assay was *Prevotella* spp. which is the predominant genus in weaned pigs.[51,52] The *Prevotella*-dominant enterotype-like group in pigs is associated with increased body weight and average daily gain, which is attributed to better energy gain from the plant-based diet.[6] A *Prevotella* dominant enterotype is also present in humans and is associated with a healthier diet (high fibre/low fat); however, the role of the different species and strains of this genus in human health and disease is controversial.[53] The addition of 5kDaR and CH had no effect on *Prevotella* spp. counts, whereas YBG reduced its counts. Despite being saccharolytic bacteria, variation in the repertoire of polysaccharide-degrading enzymes exist among the different *Prevotella* spp. members.[8,54] This might lead to a limited ability to utilise YBG as observed for oat β-glucan[55] and, consequently, to the need to compete for nutrients with other members of the faecal microbiota, thus explaining the reduced counts.

Acetate, propionate and butyrate are the major SCFA with many health benefits to the host produced during the microbial fermentation that takes place in the GIT.[56,57]

Butyrate, in particular, is the major energy source for colonocytes and its absence is associated with energetic stress and autophagy, which is observed in the colonic epithelium of germ-free mice.[58] Furthermore, butyrate, as a histone deacetylase inhibitor, interferes with the expression of pro-inflammatory cytokines exerting anti-inflammatory activity in mice and suckling pigs.[59,60] The addition of 5kDaR and CH increased butyrate concentration. Dietary supplementation of weaned and growing pigs with casein-derived peptides was also associated with an increase in butyrate production.[49,50] YBG, contrarily, decreased butyrate concentration. This is probably linked with the reduction of certain members of the microbiota due to YBG supplementation. *Prevotella* genus, for instance, has previously been correlated with increased butyrate production,[61] most likely *via* bacterial cross-feeding interactions.

It was indicated that these bioactives affect the faecal microbiota in the batch fermentation assay. Thus, the direct effects of these compounds on the growth of selected *Lactobacillus* spp. and *Bifidobacterium* spp. strains with known benefits to human and animal health were evaluated. Human and pig isolates of *L. plantarum*, *L. reuteri* and *B. thermophilum* confer colonisation resistance against various intestinal pathogens including *S. typhimurium* and pathogenic *E. coli* strains and reduce disease incidence through competitive exclusion and the production of organic acids and bacteriocins.[62–74] These strains have also been associated with beneficial changes in the composition of the microbiota, immunomodulation and maintenance of intestinal integrity.[70,75–79] The addition of 5kDaR had the most pronounced effect, as it increased *B. thermophilum* and both *Lactobacillus* spp. strains consistent with the results from the batch fermentation assay. Thus, 5kDaR seems to be capable of directly stimulating the growth of health-promoting genera in the mammalian gut. CH solely increased *L. plantarum*, indicating variability within the lactobacilli population with regard to utilising this compound. Casein-derived and cheese-originating peptides have previously been shown to enhance the growth of various members of the *Lactobacillus* and *Bifidobacterium* genera which is in agreement with the observations of this study.[23,25,26] YBG slightly increased *L. plantarum* and *L. reuteri* counts with no effect on *B. thermophilum* counts. The absence of any effect of YBG on *B. thermophilum* counts might be attributed to the inability of this bacterium to utilise this polysaccharide.[80]

The pathogens that were included in the pure culture growth assays of this study were *S. typhimurium* and ETEC O149 of the *Enterobacteriaceae* family. *S. typhimurium* causes foodborne disease in humans, with pigs and their meat products being the major source of human infection.[81] Most pigs are asymptomatic carriers of *S. typhimurium*; however, in immunocompromised animals such weaned pigs, *S. typhimurium* infection can cause intestinal disease.[81,82] The pathogenic strain, ETEC O149, is the predominant cause of post-weaning diarrhoea in weaned pigs characterised by increased mortality, diarrhoea and stunted growth.[83] The addition of 5kDaR inhibited *S. typhimurium* growth. A casein-derived peptide with antibacterial activity against *S. typhimurium* has been identified previously.[21] Most milk-derived peptides exert their antibacterial activity by interfering with the functionality and permeability of the bacterial cell membrane.[84] YBG led to a slight reduction of the ETEC counts. The potential of dietary YBG supplementation to control ETEC infection in weaned pigs has been reported previously.[85] However, the lower faecal ETEC counts and reduced duration and severity of diarrhoea observed in that study were attributed to a more controlled inflammatory response primed by the YBG supplementation, rather than the direct effect indicated in this study.[85,86]

Despite the beneficial properties of 5kDaR that have been identified in this and previous studies,[29–31] the production of this compound is expensive. The parent CH was considered as a potential alternative; however, it had no or less pronounced effects on the various bacterial populations and strains that were included in the assays of this study. Butyrate concentration was the only exception as CH stimulated its production to a greater extent compared to 5kDaR.

Conclusions

The addition of 5kDaR in the batch fermentation and pure culture growth assays was consistently associated with increases in beneficial bacterial groups such as lactobacilli and *B. thermophilum* and a reduction in *S. typhimurium*. 5kDaR also stimulated the production of butyrate in the batch fermentation assay, a major energy source and immunomodulatory molecule of the intestinal epithelium. The findings of this study provide additional information regarding the mode of action of this bioactive within the gastrointestinal environment. Therefore, further *in vivo* nutritional studies are required on the 5kDaR potential to alleviate the negative impact of inflammatory diseases and disorders on the composition and metabolism of the mammalian gastrointestinal microbiota such as the weaning stress in pigs.

A major drawback of the 5kDaR is that it requires an expensive production process that could be overcome *via* the use of the parent CH. In this study, CH stimulated butyrate production. Despite CH being a less effective bioactive than 5kDaR in these *in vitro* studies, its digestion during the transit from the upper GIT could result in the release of the more effective 5kDaR. Thus, further research should be considered regarding the use of CH as a dietary supplement.

Author contributions

Conceptualization, T. S. and J. V. O'D.; methodology, B. V., M. J. M., A. M., M. R. and T. S.; formal analysis, B. V. and J. V. O'D.; investigation, B. V. and C. K.; data curation, B. V.; writing—original draft preparation, B. V.; writing—review and editing, B. V., J. V. O'D, M. J. M., A. M., C. K., M. R. and T. S.; funding acquisition, T. S. and J. V. O'D. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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