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Investigating the potential of different dietary fibers to stimulate butyrate production *in vitro*

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Investigating the potential of different dietary fibers to stimulate butyrate production *in vitro*

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Department of Animal Nutrition and Management

Abstract

The project aimed to evaluate the potential of dietary fiber types to stimulate butyrate production using an *in vitro* system simulating the gut of pigs. Fecal inoculum from suckling piglets were collected in two different periods and were used as inoculum to ferment ß-glucan, inulin and sucrose in two separated *in vitro* fermentation trials. Gas production profiles, pH, and short chain fatty acids (SCFAs) were measured from samples collected from the *in vitro* reactors. In addition qPCR were used in an attempt to quantify butyrate producing bacteria. Samples were taken at 6 and 24h for *in vitro* trial I, and 0, 6, 24, 48h for *in vitro* trial II. The inoculum were kept overnight in freezer or in refrigerator storage to test the effect of inoculum storage in the first experiment. The results were compared with fresh fecal inoculum sampled 2h prior to the *in vitro* trial. Gas production from both *in vitro* trial I and II showed that the replicates had a high similarity for all substrates, ß-glucan showed higher gas production than the other substrate types at the beginning of both fermentation trial I and II, while negative controls did not produce any gas. The pH were relatively stable over time in chambers with ß-glucan, while pH values for inulin and sucrose were reduced over time in both experiments I and II. All substrates resulted in higher SCFA levels after 24 and 48h. ß-glucan substrate induced an increase in SCFAs earlier than the other substrates tested. Propionate, and acetate, were dominant during the whole incubation time. *In vitro* trial II produced greater amount of SCFAs compared to *in vitro* trial I. The study did not demonstrate any difference in butyrate production between substrates tested. The results from second *in vitro* qPCR run showed the decreasing of proportion of Cq values in 10×dilution DNA samples which can justify an absolute increase in butyrate producing bacteria upon ß-glucan addition (r^2= 0.9982). Results from this study also showed an increase in Cq value, i.e. reduction of butyrate producers at 48h of incubation. In addition, the overall performance of the PCR assay did not have a good efficiency. In conclusion the research did not demonstrate any difference in butyrate production between the substrates tested.

Keywords: Dietary fibers, gut health, butyrate producers, *in vitro* fermentation, suckling pigs, SCFAs, qPCR.

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Abbreviations

BG	ß-glucan
BG-fresh	ß-glucan fresh
BG-ref.	ß-glucan refrigerated
Cq	quantification cycle
DF	Dietary fiber
DNA	Deoxyribonucleic acid
GI	Gastrointestinal
Inu.	Inulin
Inu-fresh	Inulin fresh
Inu-ref.	Inulin refrigerated
mRNA	messenger Ribonucleic acid
qPCR	quantitative polymerase chain reaction
SCFAs	Short chain fatty acids
Sucr-fresh	Sucrose fresh
Sucr-ref.	Sucrose refrigerated

1 Introduction

Dietary fibers (DF) may affect the bacterial community structure and metabolism in the porcine gut and can therefore influence animal health and performance (Metzler-Zebeli *et al.* 2013). However, it is crucial to have information on the different types of DF and their roles in optimizing gut health of monogastric animals. The gastrointestinal (GI) tract of pigs contains a diverse microbial population in the large intestine, the vast majority of which are strictly anaerobic bacteria, primarily belonging to the phyla firmicutes and bacteroidetes (Isaacson & Kim 2012). *In vitro* systems have been used to simulate intestinal fermentation in order to evaluate the fermentation profiles from different dietary fibers. The potential of microbial breakdown of dietary fibers is influenced by the degree of lignification and the nature of the carbohydrate polymers present (Knudsen *et al.* 1991). The dietary fermentation produce several fermentation products, for instance short chain fatty acids SCFAs (Acetic acid; Lactic acid; Propionic acid; Iso butyric acid; n-butyric acid; Iso-valeric acid; n-valeric acid, etc), branched chain fatty acids (BCFAs), lactate, ammonia, indoles, phenols, and various gases such as carbon dioxide (CO2), hydrogen (H2), and methane (CH4) (de Leeuw *et al.* 2008). Ammonia is produced from the deamination of amino acids and hydrolysis of urea whereas phenols are produced due to carboxylation of amino acids (Jha & Francisco Diaz Berrocoso 2016). The SCFAs, such as propionate, acetate, and butyrate are important metabolites in maintaining homeostasis on the gut microbiota and intestinal epithelial cells of pigs (Parada Venegas *et al.* 2019). Butyrate is a major energy source for colonic epithelial cells, and plays a big role in regulation of microbial homeostasis, antitumor properties, and maintains the health of the epithelial cells lining the gut (Wu *et al.* 2018).

In animal nutrition, the interest of using DFs in pig diets has increased due to an economical point of view and animal welfare perspective. Feed additives and by-products rich in fiber can be used in animal diets to optimize gut health (Jha *et al.* 2019).

The overall aim of the project was to find dietary supplements with potential to contribute to an improved pig performance by improving the resilience against enteric infections and development of the intestinal immune system. The objective of the study was to evaluate the potential of dietary fiber types to stimulate butyrate production using an *in vitro* system simulating the large intestine of pigs. The laboratorial experiments screened three substrate types and one control without fiber supplement, to see which one that have the best capacity for stimulating butyrate production or the bacteria involved in butyrate production. The research questions were: 1. to evaluate how different prebiotic fibers influence gas production, pH, and SCFAs *in vitro*; 2. to investigate if ß-glucan and inulin influence levels of butyrate producing bacteria; 3. to compare how the handling of fecal samples prior to *in vitro* fermentation influence on the performance of the *in vitro* system. The results of the study can contribute to identify specific soluble fibers that through supplementation during suckling stimulate intestinal fermentation and enhance levels of butyrate producing bacteria and butyrate in the gut, which serve as energy source for the gut epithelium as well as a trigger for intestinal immune system maturation, thus contributing to a better gut health.

2 Literature review

2.1 Dietary fiber-Definition

Dietary fibers (DF) are carbohydrate-based plant materials that are neither digested nor absorbed in the upper parts of the digestive system (Capuano 2017). These are carbohydrate polymers built up by several monomeric units resistant to degradation by endogenous mammalian enzymes in small intestine (Theander *et al.* 1989). The chemical definition of DF can be described as the sum of non-starch polysaccharides (NSP) and lignin which are the main compounds of plant cell walls (Theander *et al.* 1994).

In addition, DF is made of either non-starch polysaccharides, resistant starch (RS), oligosaccharides (e.g. inulin) and non-digestible oligosaccharides (NDO), or the non-carbohydrate polyphenolic ether lignin. The NSP is made by cellulose, hemicelluloses, pectins and fructans (Knudsen 2001). Resistant starch and non-digestible oligosaccharides could be the components of DF even if they are not part of the cell wall structure, but have similar physiological effects as NSP and lignin (de Leeuw *et al.* 2008). According to their solubility in water, DF are basically classified as two physiochemical groups: the insoluble and soluble fibers (Jha & Berrocoso 2015).

Insoluble fiber is made up of substances which do not dissolve in water (cellulose, hemicellulose, lignin, and resistant starch). It can be classed as non-fermentable because of its components resist the action of intestinal micro-organisms. Soluble fibers known as fermentable fibers, is composed of water-soluble elements with a gel-forming capacity such as inulin, pectins, gums, and fructo-oligosaccharides (Jha & Berrocoso 2015).

2.2 Dietary fiber sources

Most of feed ingredients used in pig diets that includes DF have a botanical origin. Dietary fibers are found in cereals, fruits, vegetables, and legumes. Dietary fiber content and composition varies depending on plant source and food processing as well as feedstuffs produced from by-products (Knudsen 2001). The soluble DF are abundant in e.g. legumes, cereals, rye and some fruits. Pectins and xyloglucans (XG) are presented in fruits and vegetables, while arabinoxylans (AX) and mixed-linkage glucans (MLG) are the predominant non-cellulosic polymers in cereal cell walls (Selvendran 1984). β-glucan is a soluble fiber found in primarily oat and barley.

2.3 Dietary fiber in pig diets-economical and welfare perspective

In animal nutrition, the interest of using DF in pig diets has increased due to an economical point of view and animal welfare perspective. The research of Krogh *and coworkers*, 2015 showed that a high fiber diet, with a crude fiber content > 7% during the sow's gestation period can increase reproductive and growth performance, increase sow feed intake during lactation and reduce constipation and stereotypic behaviours. Dietary fiber can be used as prebiotic for serving as nourishment to stimulate the activity and growth of good bacteria in the gut (Licht *et al.* 2012). Fiber is useful in pet foods to help with weight management, diabetes mellitus, diarrhoea and constipation (Gibson 2004). The presence of fructo-oligosaccharides (FOS) in the diet selectively stimulate specific bacteria in the colon and could improve digestive health by enhancing resistance against intestinal disorders in human and pets (Gibson 2004).

The high mortality and low growth performance of piglets during the weaning can seriously affects production efficiency in the pig industry. In addition, stress caused by inadequate feed quality and the immaturity of the digestive tract and the immune system results in a decrease in feed intake and digestive disorders of piglets (Heo *et al.* 2013). However, supplementation of animal feed with β-glucan from higher plant, algae, fungi, yeasts and several other bacterial species has been shown to modulate the immune system and to influence growth characteristics of farmed animals (Vetvicka & Oliveira 2014). It has been shown to reduce levels of cholesterol, potentially through affecting the composition of the gut microbiota. (Park *et al.* 2018). Dietary fiber from feedstuffs by-products are potential sources of energy that could be used to improve animal welfare and reduce abnormalities such as stereotypic behaviors (de Leeuw *et al.* 2008).

2.4 Microbiota colonization in the gastrointestinal tract of pigs

The gastrointestinal (GI) tract of pigs is colonized by a diverse population of anaerobic and facultative anaerobic bacterial species (Banino 2012). The numbers of bacterial species distributed in the different GI sites depends in part on the different microenvironments in the GI tract. The ileum with a neutral pH and slower feed passage rate compared with stomach, hosts a large number and variety of bacteria including *Lactobacillus*, *Streptococcus*, various clostridia and *Eubacterium* as the most common groups. *Escherichia coli* and *Bacteroides* has also been found in ileum (Banino 2012).

The dominant microbial groups of caecum and colon are *Bacteroides*, *Prevotella*, different clostridia, *Lactobacillus*, *Streptococcus*, *Megasphera*, *Selenomonas*, *Mitsoukella*, *Fusobacterium* and *Eubacterium* (Banino 2012). The caecum and colon host both higher number of bacteria and microbial diversity compared with stomach (Hillman *et al.* 2017). The high number of various bacteria is due to slower feed passage rate and the anti-peristaltic movements in the large intestine that contribute to a favourable environment for bacterial growth (Hillman *et al.* 2017).

2.5 Effect of fermentation of dietary fibers in gastrointestinal tract of pigs

Dietary fibers have diverse nutritional benefits resulting from effects in both the small and large intestines of pigs. DF is indigestible by mammalian enzymes. However, various components are fermentable by the complex microbiota within the GI tract (Cummings *et al.* 2009). In the large intestine, the major end-products of gut microbiota resulted from DF fermentation are SCFAs principally acetate, propionate, and n-butyrate, also known as volatile fatty acids (Knudsen *et al.* 1991) which play an important role in the maintenance of colonic homeostasis, a crucial balance between the host, its immune system, and the gastrointestinal microbial partners (Holmes *et al.* 2012). In pigs, the largest concentrations of SCFAs are obtained in cecum compared with what is found in the colon and rectum (Tsukahara *et al.* 2003). SCFAs are rapidly absorbed from the intestine and utilized by the host as substrate for metabolic energy production. The colonocytes are dependent on SCFAs as source of energy. (Nakatani *et al.* 2018).

The absorption of SCFAs from the lumen can easily cross the gut epithelium and interact with surface molecules on the immune cells in the lamina propria into the blood of piglets. This absorption mechanism is associated by mucosal gene expression such as monocarboxylate transporter 1 and occludin (Nakatani *et al.* 2018). In addition, the mechanisms involved in SCFA absorption is influenced by the major SCFA-receptors G-protein coupled receptors (GPRs) such as GPR41 and

GPR43 able to coordinate several signaling pathways and regulate gene expression in immune cells (Luu & Visekruna 2019). In the longer term, SCFAs have also been related to protection against inflammatory bowel diseases, such as ulcerative colitis, as well as protection against colorectal cancers (Holmes *et al.* 2012). SCFAs regulate leukocyte functions including production of cytokines such as TNF-α (tumour necrosis factor-α), IL-2 (interleukin-2), IL-6, IL-10); eicosanoids and chemokines e.g., MCP-1 (macrophage chemoattractant protein-1) and CINC-2 (cytokine induce neutrophil chemoattractant-2) (Vinolo *et al.* 2011).

2.6 Overview of butyrate production in pigs

Butyrate is one of the dominant SCFAs produced by intestinal microbial fermentation of dietary fibers (Hamer *et al.* 2008). The recent research of Zhao *et al.* (2018) stated that butyrate is synthesized via pyruvate and acetyl-coenzyme A (CoA), by the breakdown of complex polysaccharides that escape digestion in the upper gastrointestinal tract and reach the colon. It has been highlighted as important due to its anti-inflammatory properties and plays an important role in the maintenance of colonic homeostasis (Tedelind *et al.* 2007). Butyrate has been shown to be one preferred energy source for colonocytes and is absorbed and used by the colonic epithelium (Scheppach 1994). Butyrate has also been implicated in down-regulation of bacterial virulence, both by direct effects on virulence gene expression and by acting on cell proliferation of the host cells (Scheppach 1994).

Butyrate can improve the barrier function of the colonocytes by increasing the secretion of antimicrobial peptides and mucus as well as the expression of tight junction proteins, thickening and strengthening the barrier while making it less hospitable to invasive microbes (Campbell *et al.* 2012). The immunomodulatory activities of butyrate result in anti-inflammatory effects, including differentiation into regulatory T-cells (Arpaia *et al.* 2013); the limitation of pro-inflammatory CD4+ T cell activity (Fontenelle & Gilbert 2012); the stimulation of epithelial cells to produce retinoic acid (Schilderink *et al.* 2016); and the desensitization of colonic epithelial cells to gamma interferons (IFN-γ) (Zimmerman *et al.* 2012).

2.7 Butyrate producing bacterial community in gastrointestinal tract of pigs

Butyrate producing bacteria are important for a healthy colon and can be found in several parts of the GIT, but they are primarily enriched in the large intestine in a monogastric animal (Vital *et al.* 2014). There are several bacterial genes that code for the enzymes involved in butyrate production where the butyryl coenzyme A (CoA):acetate-CoA transferase (EC 2.3.8.3) has been identified as a gene of primary importance for butyrate production in intestinal ecosystems (Trachsel *et al.* 2016). In both humans and animals, members of clostridia cluster IV and XIVa are the primary producers of butyrate in the gut (Miquel *et al.* 2014; Miyake *et al.* 2015).

The previous research of Vital *et al.* (2014) has been done by screening human sequenced bacterial genomes from the Integrated Microbial Genome database stated that Firmicutes, Actinobacteria, Bacteroidetes, Fusobacteria, Proteobacteria, Spirochaetes, and Thermotogae are potential butyrate producers according to the genes they express, including those that encode enzymes that synthesize butyrate, such as butyryl-CoA dehydrogenase, butyryl-CoA transferase and/or butyrate kinase. In addition, the production of other SCFAs such as acetate and lactate is mediated by bacteria such as *Bifidobacterium* species (belonging to the Phylum Actinobacteria) during carbohydrate fermentation, while the mucin-degrading bacteria *Akkermansia muciniphila* (Phylum *Verrucomicrobia*) produces both propionate and acetate (Louis & Flint 2017).

3 Materials and methods

3.1 Experimental design and sample collection

Fecal samples from 6-7 weeks old-suckling piglets at Swedish Livestock Research Center were collected in tubes and used for two separate *in vitro* fermentation experiments, where three different substrates were evaluated based on measurements of gas production, pH and SCFA. Moreover, samples from the second *in vitro* trial together with samples from an earlier performed chicken *in vitro* trial were used for quantitative PCR (qPCR) analysis to evaluate if there were differences in levels of butyrate producing bacteria. The study was performed at the laboratory of Department of Animal Nutrition and Management, SLU, Uppsala.

3.2 Materials and substrates used for the *in vitro* fermentations

The experiment was carried using stool samples from 6-7 weeks-old piglets as inoculum from the *in vitro* trial. In the *in vitro* trials three different substrates were used: ß-glucan from oats (Swedish Oat Products, Bua, Sweden), inulin (Beneo, Mannheim, Germany) and sucrose (positive control). In addition, samples from an earlier performed *in vitro* trial using chicken stool samples and with inulin (Beneo) as substrate were also included in some of the analyses.

3.3 *In vitro* fermentation experiment I: Preparation of Inoculum and substrates.

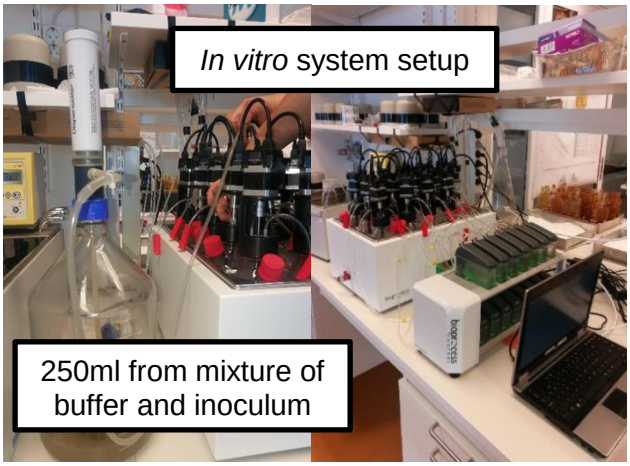

Figure 1. In vitro system set up. (Photo: Pierre Celestin, SLU)

The fecal inoculum from piglets were collected in four separate tubes at Swedish Livestock Research Center and transported to the laboratory. Two tubes were kept overnight in refrigerator at 4˚C, while the remaining two tubes were kept overnight in the freezer at -80°C. The VOS buffer was used for the *in vitro* trials and was prepared according to Lindgren 1979. In brief, four liters of the VOS buffer was prepared by using the following ingredients (g/l): K_2HPO_4 23.2g; $(NH_4)2HPO_4$ 2g; NaCl 4g; $MgSO_4$ x 7 H_2O 2g; $FeSO_4$ x 7 H_2O 0.04g; $CaCl_2$ 0.04g; and 4000ml boiled deionized water. The buffer pH was in the range 6.9-7.1 and was prepared the day before the experiment. In addition, 8.5g/l of bicarbonate ($NaHCO_3$) was added within 2h prior to the *in vitro* trial. The prepared buffer was divided into two different flasks. One buffer flask was mixed with 40 g (20g/l buffer) of fecal samples that had been stored overnight in refrigerator, while the second flask was mixed with 40 g (20g/l buffer) of fecal samples that had been stored overnight at -80°C. The mixture in each flask were homogenized and filtered using cheesecloths (to remove particles) into new 2000ml flasks. The buffers were constantly bubbled with CO_2 in order to create and anaerobic environment.

3.4 *In vitro* fermentation procedures and cumulative gas production

The *in vitro* system used were a Gas Endeavour system (Bioprocess Control, Lund, Sweden). Twelve 500ml glass bottles were used in the first *in vitro* trial (six bottles for refrigerated inoculum and separate six for frozen inoculum). In each glass bottle 5g of substrate were added (either beta glucan, inulin or sucrose). Duplicates were used for each substrate. The bottles were fixed in a thermostatic water bath at 38°C.

Then, 250ml volume of the filtered mixture of refrigerated fecal inoculum and buffer were added into the bottles labelled ‘’ref’’. In addition, 250ml of the buffer and inoculum was added to an empty bottle which served as a negative control. The procedure were then carried out with the filtered mixture deriving from the frozen fecal inoculum and buffer, where 250ml were added to each bottle labelled ‘’froz.’’ and again 250ml were added to an empty bottle that served as negative control. All bottles in water bath were closed and connected to the flow cells unit (FCU) of gas volume measuring device (*in vitro* system I). Then the *in vitro* system was started and the fermentation was runned for 24h.

Figure 2. Sample collection in different time points. (Photo: Pierre Celestin, SLU).

3.4.1 Sampling time points

Sampling from the *in vitro* system was carried out after 6h and 24h. At each sample time point, 5ml of samples were collected from each glass bottle using syringes where 1,5ml of the solution was added to a tube for SCFA analysis, 1,5ml added to a tube for DNA extraction; while the 2ml of samples left from syringe were collected for pH measurements. The pH sample tubes were taken immediately for pH measurements, while DNA and SCFA samples tubes were stored in the freezer at -20°C until further analysis. The data from the *in vitro* fermentation computer was saved and taken for cumulative gas production profile analysis.

3.5 *In vitro* fermentation experiment II

The second *in vitro* fermentation experiment was carried out with a similar procedure as the first one and were conducted by using the same substrates, but with the difference that 20g/l of fresh inoculum sampled 2h prior to the *in vitro* trial were used as inoculum. Sampling time points in the *in vitro* fermentation were 6, 24 and 48h and aliquots were taken for analysis of pH, SCFAs and DNA isolation. Aliquots for DNA isolation were also taken prior to the *in vitro* fermentation at 0h. The *in vitro* experiment was carried out for 48h and each substrate were analysed in duplicates. Moreover, two glass bottles without any substrate added were included as negative controls.

3.6 Short chain fatty acids (SCFAs)

Collected *in vitro* samples were centrifuged for 5 minutes at 13000 rpm. The aliquot solution was then analyzed directly by the high-performance liquid chromatography (HPLC) according to the method described below (Andersson & Hedlund 1983).
The amount of acetate, butyrate, and propionate were determined by HPLC. The HPLC-system used consisted of Alliance 2795 Separations Module with Temperature control Module II range 0-150ºC and 2414 RI Detector (Waters Assoc. USA). A ReproGel Column (300 * 8mm) with a puticel size of 9µm, were used as the separation column and an ReproGel H, 9µ * 30 * 8 mm (Dr.A.Maisch, Ammerbuch, Germany) was used as a pre-column. The conditions used for the HPLC analysis were: Mobile phase of 5mM H_2SO_4; flow rate of 0.8 ml/min; column temperature at 60ºC; and Injection volume of 20µl.
Calibration: The instrument was calibrated by injecting solutions containing known amounts of the analysed organic acids. The calibration was performed using peak-height method.

3.7 Butyrate producing bacteria quantification by real-time PCR (qPCR)

3.7.1 DNA Extraction

DNA was extracted from the second *in vitro* experiment trial and from the ß-glucan fermentation samples only. In addition, samples from an earlier *in vitro* trial where inulin had been fermented with cecal digesta from chickens were also used for DNA extraction. The inulin samples from the earlier *in vitro* fermentation was selected based on the criteria that they had already shown to stimulate butyrate production. The avian inulin samples were extracted from frozen samples collected after 6, 12, and 24h of fermentation with inulin.

Total genomic DNA was extracted from collected 1ml aliquots using the QIAamp fast DNA Stool Mini Kit according to the protocol from the manufacturer. The DNA concentrations were measured by a Qubit 3.0 fluorometer (ThermoFisher Scientific).

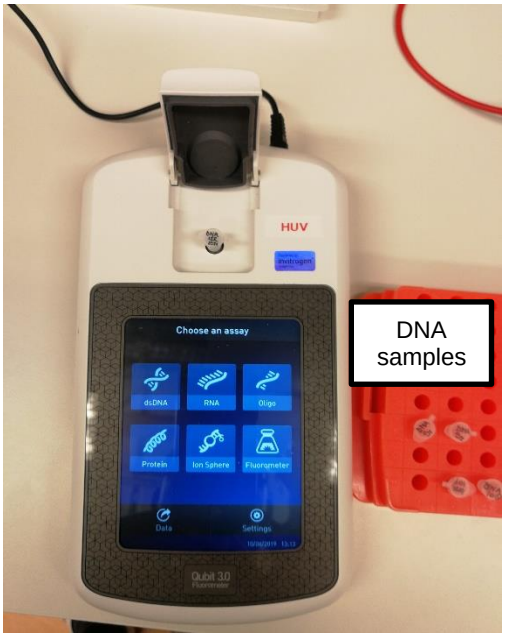

Figure 3. Qubit for measurements of DNA concentrations

Table 1. *DNA Sample codes and conc. measured by Qubit assays with Qubit 3.0 fluorometer*

Sample code	Concentration (ng/ µl)
BG 0h	26.7
BG6h	150
BG24h	222
BG48h	64.6
10N6h	6.7
10P12h	11.7
10Q24h	15.4

BG 0h, BG6h, BG24h, BG48h (Beta glucan sample collected at 0, 6, 24, 48hours of porcine *in vitro* trial II). 10N6h, 10P12h, 10Q24h (Inulin sample collected at 6, 12, 24hours of avian *in vitro* trial).

3.7.2 Quantitative PCR

The seven DNA samples (**Table 1)** were analyzed for the presence of butyrate producing bacteria by real-time quantitative PCR (qPCR). To quantify the butyrate producers, the primer pair (funbut-FWD, 5′-CARYTIGGIATYGGIGGIATSCC;

funbut-REV, 5′-TGTCCGCCIGYICRSWRAT) were used (Trachsel *et al*. 2016) to detect and quantify the gene encoding butyryl-CoA transferase.
Each reaction was run in duplicate in a total volume of 25 µl with 2 µl DNA and 23µl Master mix, (QuantiTect SYBR Green PCR Kit; Qiagen, Hilden, Germany) in white 96-well reaction plates sealed with optical adhesive film in a qPCR machine (Bio-Rad, Hercules, CA, USA). The basic protocol used was an initial cycle of 95°C for 15 min followed by 40 cycles of 95°C for 15 s, the assay specific annealing temperature for 30 s and 72°C for 30 s. The protocol was ended with a melt curve step for product verification. Five-fold serial dilutions of a DNA sample that scored positive with this primer pair was used for optimization of annealing temperature and primer concentration. The combination finally chosen was an annealing temperature of 53ºC and a final quite high primer concentration of 1500nM. However, these conditions were still suboptimal, displaying alternate priming in concentrated samples and a PCR efficiency of at best 85%.

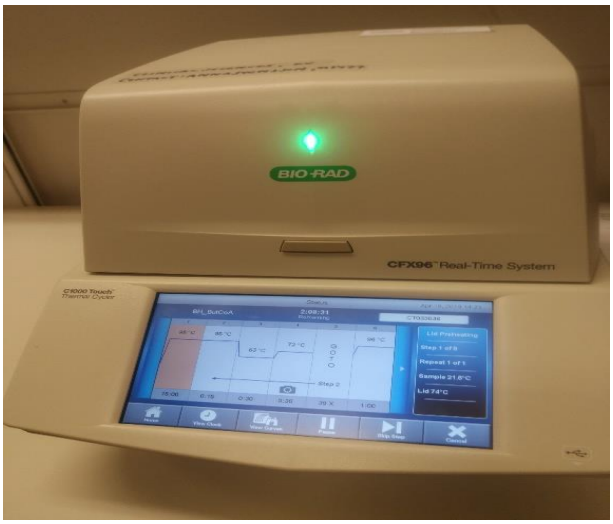

Figure 4. qPCR machine (Bio-Rad, Hercules, CA, USA)

No suitable standards were available for absolute quantification. To get a rough estimate, raw Cq values were compared between samples (diluted 10 times to minimize the occurrence of PCR artefacts), i.e. expressed as Cq values per µl. To get an estimation in relation to extracted DNA, these samples were further diluted so as to have the same concentration as the one with the lowest concentration (Table 2).

Table 2. *Concentration adjustments*

Sample code	New Conc. (µl)	Water quantity (µl)
BG6h	1.78	8.22
BG24h	1.2	8.8
BG48h	4.13	5.87
10P12h	5.72	4.28

10Q24h	4.36	5.3

The old concentration of BG 0h and 10N6h (2.67 µl; 0.67 µl respectively) were used without calculating the new concentration and water quantity for dilution.

3.8 Data analysis

The cumulative gas production kinetics, pH from fermentation points and the quantification of SCFAs levels were analysed in Past statistical software. Results from the second *in vitro* experiment with BG fresh samples; together with inulin from *in vitro* chicken samples were performed in technical duplicates in the qPCR analysis. Amplification and melting curve were determined for testing the sensitivity and precision of qPCR run. The average of Cq values (Cq value is a relative measure of concentration of target genes in the PCR reaction) were determined by calculating the mean between the two Cq values. Cq Av= (Cq1+ Cq2)/2. A linear relationship between Cq values (Y) and (X) (log quantity) were calculated by exponential equation: Y= 6E+07e-0.767x.

4 Results and Discussion

4.1 Gas production kinetics

The results from the first *in vitro* trials showed that the replicates had a high similarity in gas production profiles for all substrate types fermented in frozen and refrigerated fecal samples. BG showed higher gas production than the other substrate types at the beginning of incubation, but the gas production levelled out during the end of the experiment. The negative controls duplicates did not produce any gas for neither the frozen nor the refrigerated inoculums. The inulin duplicates fermented in frozen stool samples had lower gas production profile at the beginning which then increased at the end of incubation period, followed by inulin duplicates fermented in refrigerated stool samples which had shown the higher rate of gas production **(Fig 5)**.

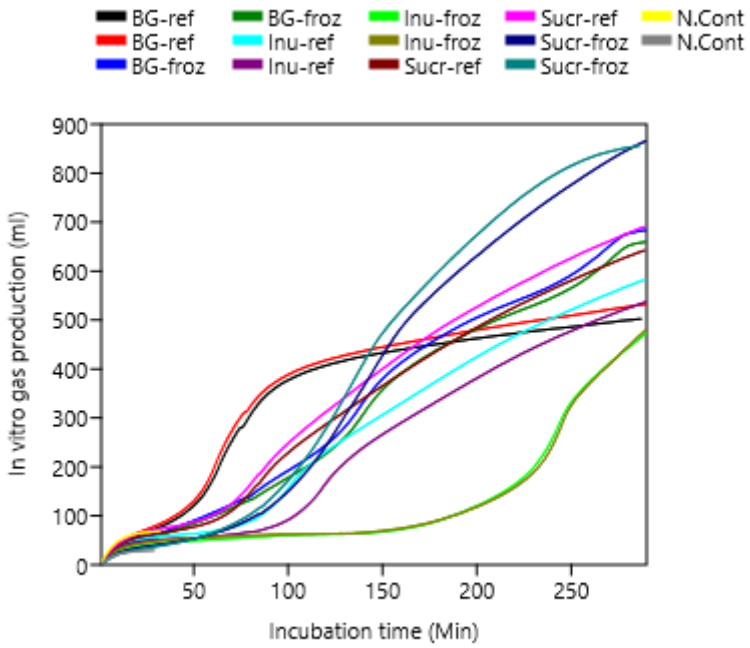

Fig 5. Cumulative gas production profiles (ml) within time (Min) from *in vitro* trial I during 24 hours of incubation period.

The fibers fermented in first *in vitro* run with samples stored in refrigerator temperature overnight produced in total smaller amount of gas compared with experiment II where fresh inoculum sampled 2h prior to the *in vitro* trial were used. The reason could be the greater microbial activity as the major factor for producing higher gas production rates (Jensen & Jørgensen 1994). The gas profile differ considerable dependent on if you froze the samples or if you stored them in the refrigerator.

Results from *in vitro* trial II did not show any differences between inulin and sucrose in gas production. They followed the same kinetics and in the first round it ended up with higher levels of gas with sucrose, whereas in trial two inulin was slightly higher (figure 6). Gas production kinetics resulted from both *in vitro* fermentation I and II showed that beta glucan had a different gas profile compared with both sucrose and inulin.

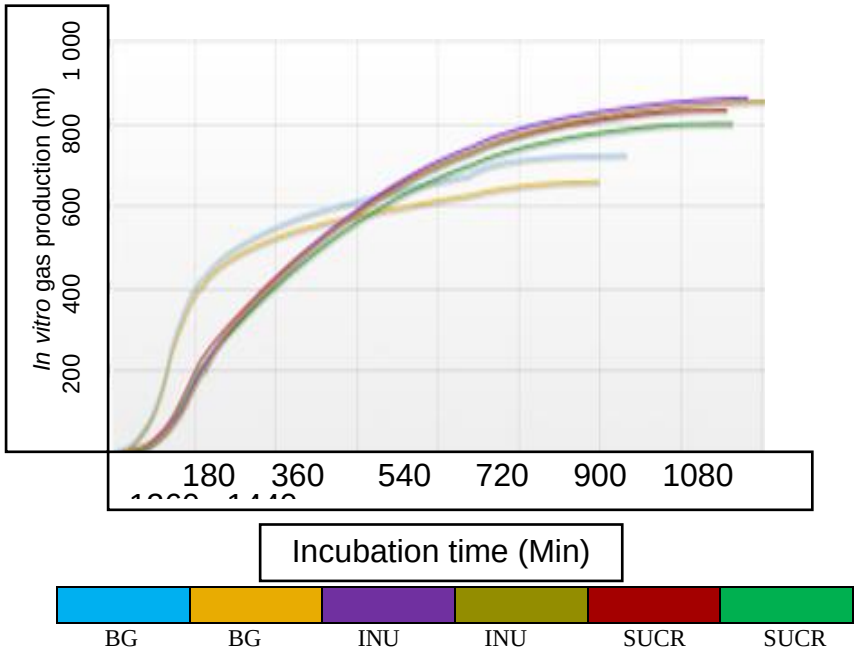

Fig 6. Cumulative gas production profiles from *in vitro* trial II with fresh fecal samples sampled 2h prior to the *in vitro* trial.

4.2 pH analysis

The pH were measured in the samples collected from both the first and second *in vitro* trials (**Fig. 7 and 8**). The pH decreased slightly for 6 h to 24 in the second *in vitro* experiment for the BG, but was otherwise stable over time in chambers with BG. The pH values for inulin and sucrose decreased over time and followed the same pattern in both experiments. The pH of negative control duplicates remained the same throughout the whole fermentation, but increased slightly after 48h.

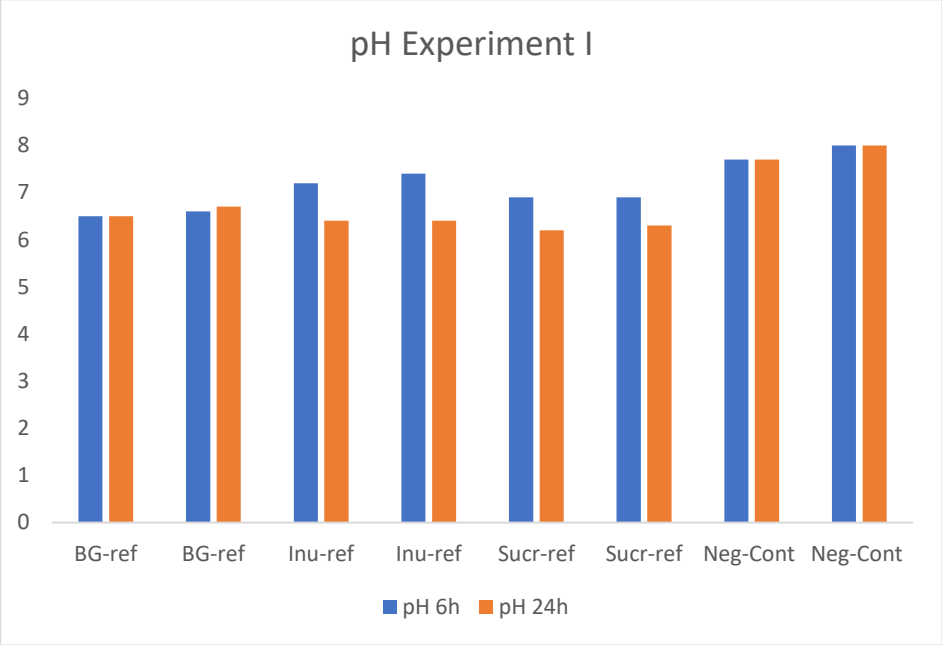

Fig 7. Measurements of pH from *in vitro* trial I with refrigerated inoculum after 6 and 24h of incubation, using a different substrates BG, Inu, Sucr and a negative control without any substrate run. BG-ref (Beta glucan refrigerated), Inu-ref (Inulin refrigerated), Sucr-ref (Sucrose refrigerated), Neg-Cont (Negative Control).

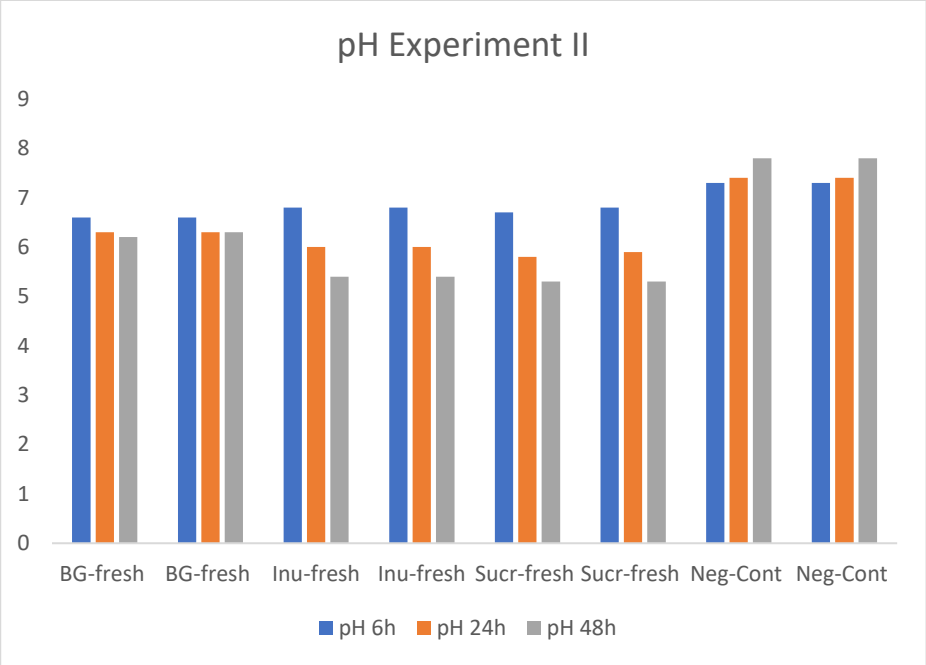

Fig 8. Measurements of pH in exp II after 6, 24, and 48h of incubation, using a different substrates BG, Inu, Sucr and a negative control without any substrate. BG-ref (Beta glucan refrigerated), Inu-ref (Inulin refrigerated), Sucr-ref (Sucrose refrigerated), Neg-Cont (Negative Control).

The study did not detect a decrease for BG in pH as for Inulin and Sucrose, but the reason for that is not known. At 6h, BG is a bit lower compared with the other substrates and in addition, the SCFA for BG is higher at 6h. This is as same as the previous report of Kim & White (2009) justifying that *in vitro* fermentation of beta-glucan lowered pH from 4 to 8h of the beginning of fermentation. The previous study showed that the low pH resulted from beta glucan fermentation in the human gut is caused by the production of SCFA which can prevent the growth of harmful bacteria and contribute in the absorption of minerals such as calcium and magnesium (Cummings 1981). In addition, the reason of pH reduction for inulin and sucrose in our study is justified by the research of Topping (1996) which has shown that the lower pH values formed as a result of the SCFA production during fermentation provide homeostasis for health of the colon.

4.3 Short chain fatty acids

Previous studies showed that the presence of carbohydrates in the colon and their fermentation might alter the colonic physiology (Williams *et al.* 2017). Dietary carbohydrates undergo fermentation in the colon of pigs and stimulate SCFAs production (Singh *et al.* 2017).
The *in vitro* fermentation trials produced different total short chain fatty acid profiles in presence of different substrate types. Results from SCFAs analysis from the *in vitro* trial I and II are shown in **Fig 9 and 10.** All substrates resulted in higher SCFA levels after 24 and 48 h. *In vitro* trial II produced greater amount of SCFAs than trial I. However, in this study, there were no difference in butyrate production between the tested substrates.

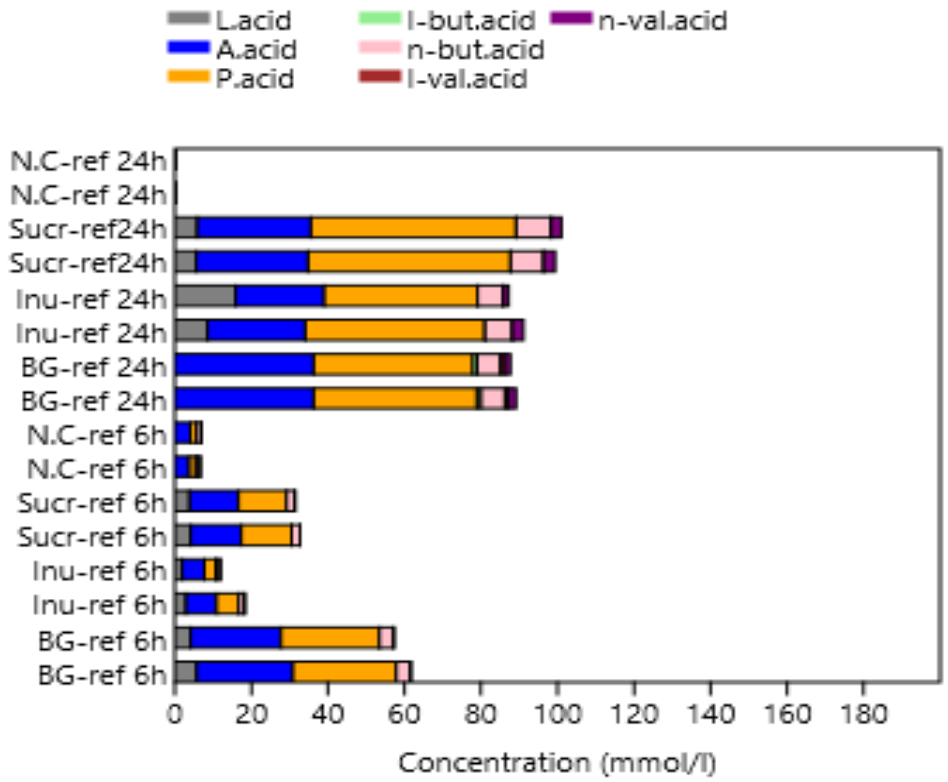

Fig 9. The total SCFA profiles from *in vitro* trial I. L.acid (Lactic acid); A.acid (Acetic acid); P.acid (Propionic acid); I-but.acid (Iso butyric acid); n-but.acid (n-butyric acid); I-val.acid (Iso-valeric acid); n-val.acid (n-valeric acid); BG (Beta glucan); Inu (Inulin); Sucr (Surose); N.C (Negative control); ref (refrigerated samples).

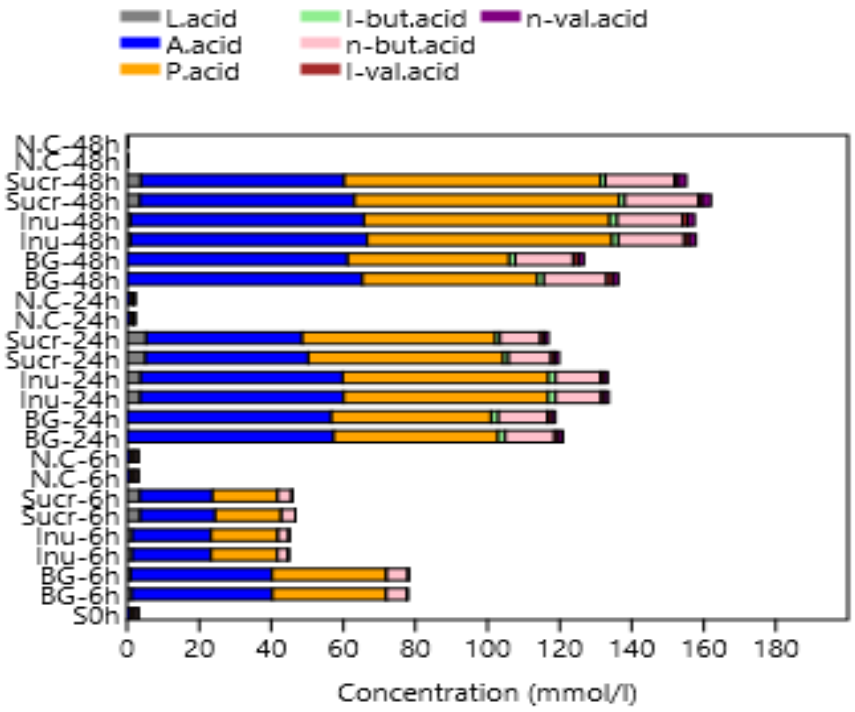

Fig 10. The total SCFA profiles from *in vitro* trial II. L.acid (Lactic acid); A.acid (Acetic acid); P.acid (Propionic acid); I-but.acid (Iso butyric acid); n-but.acid (n-butyric acid); I-val.acid (Iso-valeric acid); n-val.acid (n-valeric acid); BG (Beta glucan); Inu (Inulin); Sucr (Surose); N.C (Negative control).

BG substrate induced an increase in SCFAs earlier than the other substrates tested (**Fig 9 and 10)**. Propionic acid is the most dominant in both **Fig 9 and 10.** The propionate and acetate dominated during the whole incubation time which is in agreement with the study of den Besten *et al.* 2013. However, the proportions of the SCFA is not in agreement with how it looks in vivo, where acetate is usually more dominant (commonly 60% of the SCFA is acetate, 20% propionate, 15% butyrate and 5% others volatile fatty acids (Liu *et al.* 2012). This could partly be due to the origin of the inoculum samples used in the *in vitro* trial. Moreover, it could also be due to that the *in vitro* system can introduce biases. In vivo, the SCFA are continuously absorbed from the gut whereas *in vitro* the SCFAs accumulate and this could lead to differences in SCFA composition compared with in vivo.

Results from SCFA production were similar for Inulin and Sucrose after 24 and 48h; while SCFA production was greatest for BG at 6h. This might be justified by the evidence from previous research which has shown that the potential of microbial breakdown of dietary fibers is influenced by the degree of lignification, solubility ratio, fermentation time, and the nature of the carbohydrate polymers present (Knudsen et al. 1991).

4.4 Quantitative PCR (qPCR) and effect of fibers on butyrate producing bacteria

Initially, the primers were checked for specificity by doing regular PCR which showed a single band indicating good specificity of the assay. It was difficult to find optimal PCR conditions for these primers. Although good amplification curves was achieved **(Fig 11)**, the overall performance of the PCR assay had a low efficiency (85% at best). This is different from expected efficiency considered acceptable (90-110%). The reason for the low efficiency obtained is not certain, but could be dependent on the high degeneracy of the used PCR primers.

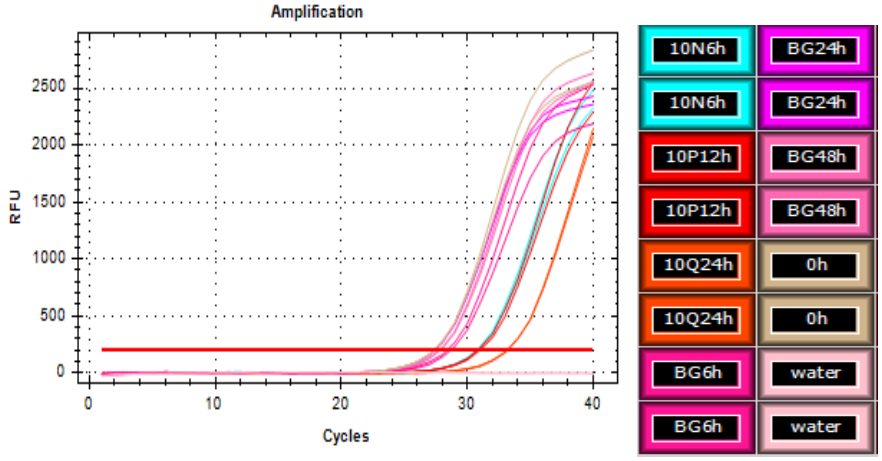

Fig 11: Amplification plot and qPCR plate layout. RFU (relative fluorescence unity).

Melting curve analysis showed two different peaks, the main peak had a Tm value which was approximately 82°C, while the second smaller peak was obtained under the threshold line with a slightly higher melt temperature 86°C (Fig 12). As negative control duplicates (water) showed no amplification at any primer concentration tested **(Fig 11, 12)** one can conclude that the additional peak is due to alternative priming on bacterial DNA. This is not so surprising given the high degeneracy of the primers and the primer concentration used.

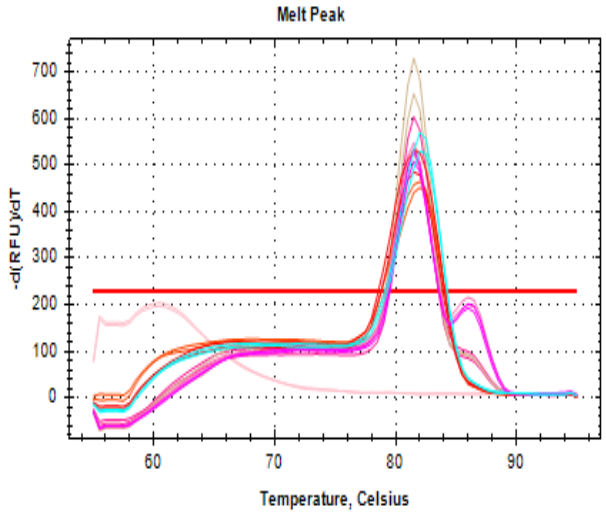

Fig 12: Melt curve of SYBR green PCR products. The Y-axis represents the derivative reporter (−Rn) while x-axis represents the temperature (°C). Two different peaks were seen, the first one at Tm values which was approximately 82°C, while the second one was obtained under the threshold line with a slightly higher melt temperature 86°C.

To get a rough estimate of butyrate producers samples were analyzed at a 1:10 dilution, regardless of actual DNA concentration. All samples showed good technical replicates (Table 3).

Table 3. *DNA from second in vitro trial samples and Cq values.*

Sample codes	Conc.	Cq Av	Cq1	Cq2
0h 1:10	2.67	22.01	21.85	22.17
BG6h 1:10	15	19.68	19.86	19.5
BG24h 1:10	22.2	19.2	19.05	19.32
BG48h 1:10	6.46	20.86	20.86	20.86
0h	26.7	17.55	17.43	17.66
BG6h	150	16.73	16.51	16.95
BG24h	222	17.33	17.29	17.37
BG48h	64.6	17.44	17.54	17.34

The table shows DNA concentrations of samples from second *in vitro* trial with ß-glucan at their dilution of 1:10. The average of Cq values was also calculated with as formula Cq Av= (Cq1+ Cq2)/2.

Assuming that the same volume of fermentation product was taken for DNA isolation and that the isolation procedure was done the same way we can get a rough idea of the presence of butyrate producers from the Cq values. From this it seems that it starts at a fairly low concentration (Cq ~22) and then increases over the next 24h (to Cq~19) to eventually decreasing again towards 48h (Fig 13).

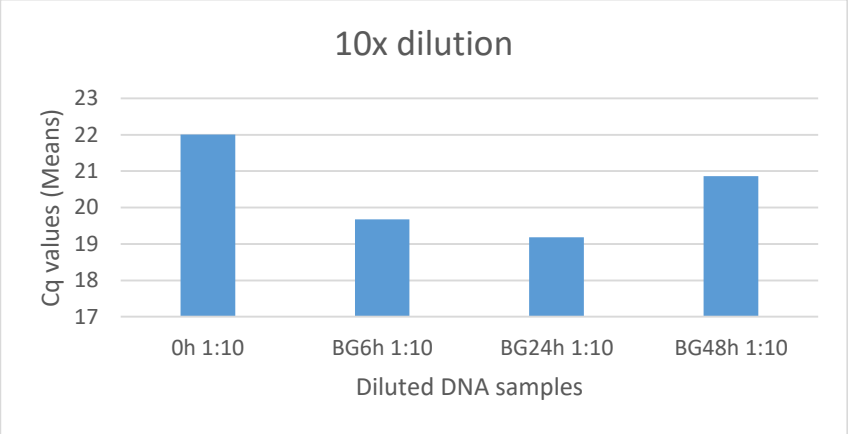

Fig 13. Raw Cq values in 10×dilution of beta glucan DNA samples. This corresponds to signal per sample volume.

To evaluate the data in relation to input DNA concentration these raw Cq values were plotted against the concentration of the samples. The result showed a linear relationship between log conc. and Cq values (R^2= 0.9982) suggesting that the **proportion** of butyrate producers do not change over time (**Fig 14).**

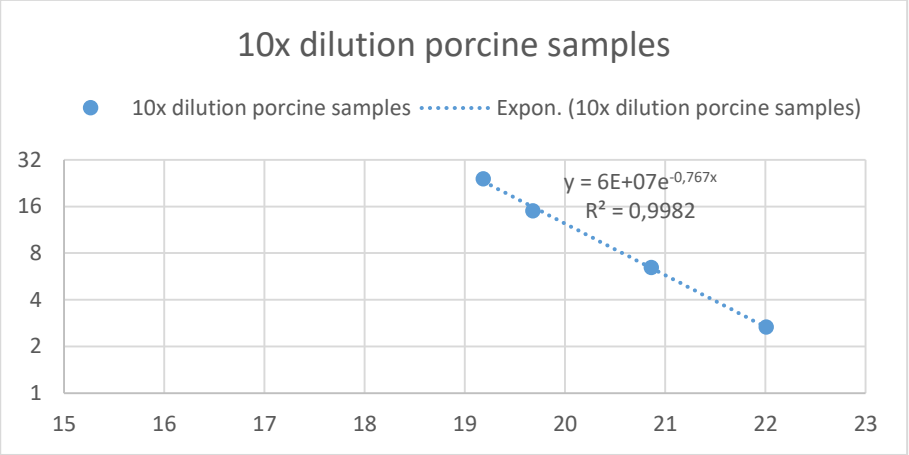

Fig 14. A scatter plot demonstrating the relationship between Cq values and DNA concentration. The function describing the relationship between Cq values and x (log quantity) were calculated by exponential equation: Y= 6E+07e-0.767x; (R^2= 0.9982).

To further analyze this the concentration of each sample was adjusted so that the same amount of input DNA was used for each sample. As seen in the amplification

plot all samples displays roughly the same Cq when the same amount of DNA is analyzed indicating that the proportion of butyrate producers remains the same (Fig 15).

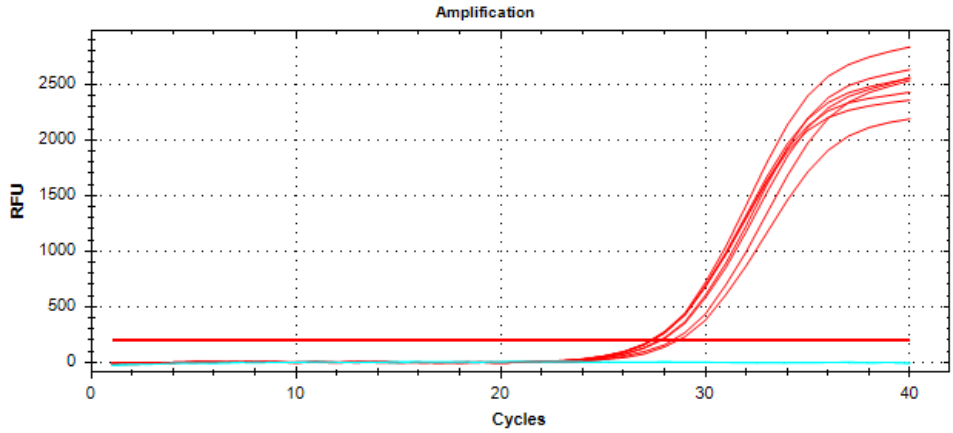

Fig 15. Amplification plot of the beta glucan DNA samples adjusted to have the same amount of input DNA. Only BG6h appear one cycle later than the other samples.

In the presented study, the effects of dietary fibres on microbial butyrate producing populations were assessed using a quantitative PCR assay targeting the butyryl-CoA transferase gene. The study showed a decrease in Cq values in DNA samples from 0 to 24h indicating an absolute increase in butyrate producing bacteria upon ß-glucan fiber addition. This has also been previously reported by the research of Fehlbaum *et al* (2018) that showed that beta glucan induced the growth of *Roseburia* (members of Firmicutes), and butyrate was significantly enriched after supplementation with beta glucan.

Results from this study also showed an increase in Cq value, i.e. reduction of butyrate producers at 48h of incubation. This is in agreement with data from the gas production that indicated that the fermentation had stopped for BG after 48h incubation, that one could assume that the bacteria started to degrade.

5 Conclusion and recommendation

This study did not demonstrate any difference in butyrate production between the substrates tested in the study. The qPCR showed that the inclusion of different dietary fibers has influenced the composition and activity of the butyrate producing community. The overall performance of the PCR assay did not have a good efficiency.

As a first step, some more trimming of the *in vitro* system are needed in future studies, since the data did not really resemble the in vivo situation. Continued research on butyrate production is needed to understand the mechanisms that influences butyrate producing bacteria in pigs.

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