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Impact of colonic fermentation of a plant sterol-enriched rye bread on gut microbiota and metabolites †

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Abstract: Studies on the impact of colonic fermentation of plant sterol (PS)-enriched foods using dynamic *in vitro* models are limited. This study aims to evaluate the effect of a 72h-dynamic *in vitro* digestion-colonic fermentation (using the simgi® system) of a PS-enriched rye bread on colonic microbial population, and short chain fatty acids (SCFA) and ammonium production. In all colon compartments (ascending colon (AC), transverse colon (TC), and descending colon (DC)) (72 *vs.* 0h), a reduction in ammonium concentration (5.7-9.4-fold) and an increase in *Staphylococcus* spp. (1.4-2.1-fold), *Lactobacillus* spp. (1.5-fold), *Bifidobacterium* spp. (1.3-1.5-fold), and *Enterococcus* spp. (1.4-1.6-fold) was observed. In AC and TC, total SCFA decreased (4- and 1.3-fold, respectively), while increased in DC 1.4-fold due to an increment of butyrate content from 4 to 45mM. These results suggest that PS-enriched rye bread favors the growth of beneficial microbial species and production of butyrate, a fuel source for the enterocyte, promoting health benefits.

Keywords: : *in vitro* colonic fermentation; plant sterols; microbiota; short chain fatty acids; ammonium

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1. Introduction

Diet is known to modify the gut microbiota, which potentially influencing the host's health through shifts in microbiota composition, diversity, and richness [1]. Wholemeal rye bread is an excellent source of fiber (arabinoxylan, fructan, cellulose, and β-glucan) [2] which can be fermented by the microbiota producing metabolites like short-chain fatty acids (SCFA, such as acetate, propionate, and butyrate). These metabolites play crucial roles like promoting gut integrity and regulating glucose homeostasis, lipid metabolism, appetite, immune system, and inflammatory response [3]. Fiber-rich food (such as wholemeal rye bread with 15.3 g fiber/100 g bread [4]) is able to selectively promote the growth of *Bifidobacterium*, a specific acetate- and butyrate-producing bacteria [5], as well as *Lactobacillus* [6].

Regarding plant-sterols (PS), it is well-known their efficacy as a cholesterol-lowering agent (reducing plasma cholesterol concentrations up to 12% with a daily intake of 1.5 to 3 g) [7], as well as antiproliferative, anti-inflammatory, antioxidant, and antidiabetic properties [8]. Although, there is a lack of research on the metabolism of these bioactive compounds by the microbiota, due to their limited absorption rate (ranging from 4 to 16%), PS reach the colon where they become susceptible to the microbiota's influence, ultimately affecting metabolites such SCFA or ammonium [9].

Therefore, for the first time, this study aims to evaluate the effect of a dynamic *in vitro* digestion and colonic fermentation of a PS-enriched wholemeal rye bread (PS-WRB) on changes in the colonic microbial population, and SCFA and ammonium production.

2. Materials and Methods

2.1. Sample and oral phase digestion

The PS-WRB optimized baking procedure and the chemical composition (% w/w, dry basis) is as stated in Makran et al. [4] (protein: 7.8 ± 0.1; ash: 2.0 ± 0.1; lipid: 4.7 ± 0.2; carbohydrate: 65.2 ± 0.6; insoluble fiber: 15.2 ± 1.8; soluble fiber: 5.1 ± 0.2). For the human oral phase, three portion of PS-WRB (81.45 ± 1.14 g) were chewed as described in Faubel et al. [10] in order to obtain respective oral bolus. An optimum ratio for in vitro digestion was determined to be 1:1 (w/w) or a 100% increase in the bolus, and the oral bolus should not be thicker than tomato or mustard paste [11].

2.2. Dynamic in vitro colonic fermentation

The simgi® system (CIAL, CSIC-UAM, Madrid, Spain) was used for dynamic gastrointestinal digestion and colonic fermentation [12] with modifications. This system includes five compartments (stomach, small intestine, ascending colon (AC), transverse colon (TC), and descending colon (DC)) with a constant 37°C temperature and controlled enzyme and NaOH/HCl flow to maintain pH at each stage (Stomach: 1.8; small intestine: 7; AC: 5.6; TC: 6.3; DC: 6.8). A gastric digestion was performed for each oral bolus in a reactor without peristaltic movements and once finished manually emptying into the small intestine due to the high viscosity of the sample generated by the bread's fiber content (15.3 g /100 g bread). Enzymes and pH solutions were added progressively. In intestinal and colonic compartments, pipes connected them, and peristaltic valve pumps were automated. Pepsin (2000 U/mL, 15 mL in 150 mM NaCl) initiated gastric phase. Small intestine phase used pancreatic juice (40 mL, including pancreatin (0.9 g/L) and oxgall dehydrated fresh bile (6 g/L)). AC, TC, and DC were filled with nutrition medium (250, 400, and 300 mL, respectively). Fecal sample from a healthy donor meeting specific criteria was used for inoculating colonic compartments with 20 mL diluted fecal sample (20%, w/v) in sodium phosphate buffer (0.1 M, pH 7) with 1 g/L sodium thioglycolate. Transfer between colonic compartments was 145 mL at 5 mL/min.

After a 9-day stabilization period, the AC of the simgi® was fed with 145 mL of gastrointestinal digesta (equivalent to 40 g of PS-WRB providing 1.3 g PS per day) at a rate flow of 5 mL/min in the first 8h. Another 145 mL of gastrointestinal digesta were incorporated into the AC at the same flow rate, and at the last 8h, 145 mL of nutritive medium was added. This process was carried out for 3 days to simulate daily 80 g PS-WRB intake through AC, TC, and DC. Fermentation liquids were collected at specific time points and stored at −20°C for further analysis.

2.3. Plate count and determination of SCFA and ammonium ion

Microbiota composition analysis and SCFA and ammonium ion determinations were conducted as described in Tamargo et al. [12].

Plate counts were conducted on general and selective media after serial dilutions of fermentation liquids in sterile saline (NaCl 0.9%). Spot seeding (10 µL in triplicate) of each dilution was done on selected media: Enterococcus agar (Enterococcus spp), BBL CHROMAgar (Staphylococcus spp), Bifidobacterium agar modified by Beerens (Bifidobacterium spp), and LAMVAB (Lactobacillus spp from feces). Plates were incubated at 37°C for 24 or 72 h, depending on the culture medium. All media, except BBL CHROMAgar, were incubated in anaerobiosis cabinet (BACTRON). Colonies counted using SC6PLUS colony counter (Stuart, UK). Results expressed as log CFU/mL.

The determination of SCFA was performed by duplicate using gas chromatography on an Agilent 6890A chromatograph equipped with an automatic injector G2613A and flame ionization detector. An DB-WAXetr column (100% polyethylene glycol, 60 m, 0.32 mm x 0.25 µm) was used, and helium served as the carrier gas at a flow rate of 1.5 mL/min. The temperature gradient consisted of the following steps: 50°C for 2 minutes, followed by a 15°C/min increase to 150°C, a 5°C/min increase to 200°C, and a 15°C/min increase to 240°C for 20 minutes, resulting in a total analysis time of 41.3 minutes.

Ammonium ion determination was carried out by duplicate with the Spectroquant Ammonium Test Kit (Merck, Germany). Serial dilutions were prepared from a 10 g/L standard ammonium solution for calibration. Fermentation liquids samples were diluted (1:10) with deionized water, and before measuring at 25°C, 5 mL of NH_4-1 and NH_4-2 reagents were added to standards and samples. The mixture was stirred between reagent addition, and absorbance measured at 690 nm.

2.4. Statistically analysis

A t-test was used to evaluate statistically significant differences ($p<0.05$) in each colon compartment between 0 and 72h for microbial growth, ammonium content and individual and total SCFA content. GraphPad Prism 9.5.1 (GraphPad Software Inc., San Diego, CA, USA) was used throughout the whole study.

3. Results

Microbial growth has been observed in each colon compartment (differences statistically significant at 72 *vs.* 0h, $p< 0.05$) of all the species listed in Table 1. *Staphylococcus* spp. in the AC has shown the highest growth, being of 2.1-fold at 72h of fermentation compared with 0h, with less growth (1.4 and 1.6-fold, respectively) in the distal compartments (TC and DC). *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterococcus* spp. have shown a 1.3- to 1.6-fold increase in all three colon compartments.

Table 1. Changes in microbial growth at 0 and 72 h of PS-WRB fermentation in the three colon compartments.

Microorganisms		**0h**	**72h**[1]
Staphylococcus spp.	AC	4.28 ± 0.01	8.98 ± 0.05
	TC	5.67 ± 0.01	7.88 ± 0.05
	DC	5.64 ± 0.06	8.77 ± 0.01
Lactobacillus spp.	AC	6.01 ± 0.02	9.23 ± 0.07
	TC	6.15 ± 0.03	9.17 ± 0.02
	DC	5.71 ± 0.03	8.67 ± 0.02
Bifidobacterium spp.	AC	6.37 ± 0.01	9.34 ± 0.02
	TC	7.25 ± 0.04	9.18 ± 0.09
	DC	7.14 ± 0.08	9.23 ± 0.12
Enterococcus spp.	AC	5.80 ± 0.03	9.44 ± 0.04
	TC	6.42 ± 0.02	9.29 ± 0.01
	DC	5.71 ± 0.01	8.86 ± 0.03

Data expressed as mean values of log CFU/mL ± standard deviation ($n=3$). [1]Statistically significant difference ($p<0.05$) between 0 and 72h in each colon compartment and genera. AC: ascending colon, TC: transverse colon, DC: descending colon.

Regarding the concentration of ammonium ion (Figure 1), at 0h, the highest content is observed in the DC (465.90 mg/L), followed by the TC (375.52 mg/L), and the AC (179.82 mg/L). After 72h of colonic fermentation of the bread digesta, a statistically significant decrease ($p<0.05$) in ammonium ion content was shown in all three colon compartments. This decrease is highest in the TC (9.4-fold), followed by the DC (8.5-fold) and AC (5.4-fold).

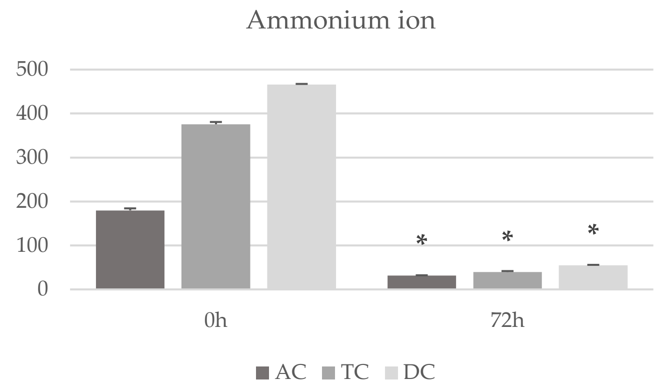

Figure 1. Ammonium ion contents (mg/L) in the colon compartments at 0 and 72 h of PS-WRB fermentation. *Statistically significant differences (p<0.05) between 0 and 72h for ammonium ion concentration for each colon compartment. AC: ascending colon, TC: transverse colon, DC: descending colon.

The main SCFA are acetate, propionate, and butyrate (Table 2). In the AC (72 *vs.* 0h), a decrease is observed for acetate and propionate (4.1- and 3.5-fold, respectively), while a slight increase (0.04 to 0.06 mM) is shown for butyrate. As a result, total SCFA (sum of acetate, propionate, and butyrate) decreases from 16.22 to 4.03 mM (Figure 2). In TC (72 *vs.* 0h), acetate remains constant without statistically significant differences (p<0.05) while propionate and butyrate decrease (4.8- and 1.2-fold, respectively). Total SCFA in TC decreases from 49.61 to 39.95 mM. In DC (72 *vs.* 0h), acetate and propionate decrease 1.3- and 1.7-fold, respectively, however, the largest increase of 11.8-fold for butyrate causes an increase in total SCFA (from 67.34 to 92.37 mM).

Table 2. Contents of short chain fatty acids (SCFA) at 0 and 72 h of PS-WRB fermentation in the three colon compartments.

SCFA		**0h**	**72h**
Acetate	AC	15.11 ± 0.02	3.66 ± 0.05*
	TC	35.25 ± 0.36	35.46 ± 0.22
	DC	40.29 ± 0.59	29.88 ± 0.25*
Propionate	AC	1.07 ± 0.03	0.31 ± 0.12*
	TC	12.03 ± 0.09	2.51 ± 0.03*
	DC	15.42 ± 0.22	9.06 ± 0.02*
Butyrate	AC	0.04 ± 0.002	0.06 ± 0.001*
	TC	2.33 ± 0.01	1.98 ± 0.17*
	DC	3.79 ± 0.11	44.89 ± 0.02*

Data expressed as mean values (mM) ± standard deviation (n=2). *Statistically significant differences (p<0.05) between 0 and 72h for each SCFA at the same colon compartment. AC: ascending colon, TC: transverse colon, DC: descending colon.

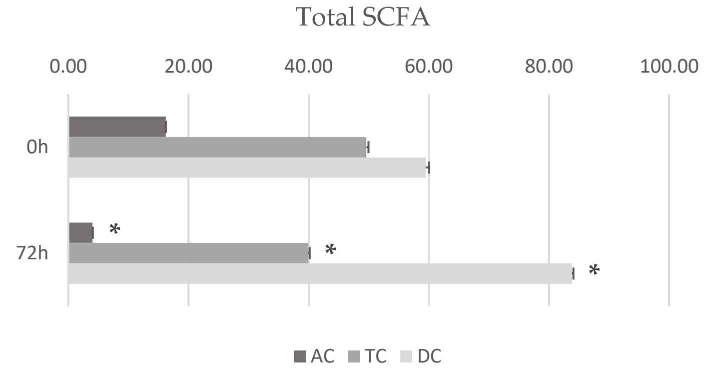

Figure 2. Total short chain fatty acids (SCFA) contents (Mm) in the colon compartments at 0 and 72 h of PS-WRB fermentation. *Statistically significant differences (p<0.05) between 0 and 72h for total SCFA for each colon compartment. AC: ascending colon, TC: transverse colon, DC: descending colon.

4. Discussion

In the present study, we evaluated the impact of a complex food matrix, such as PS-WRB, on microbiota composition and metabolite production employing a simgi® system. Colonic fermentation of PS-WRB leads to an increase in *Lactobacillus* and *Bifidobacterium* species, probably due to the bread components. In fact, the bread has an important content of fiber (15.3 g /100 g bread) and is source of β-glucan, identified as a prebiotic [2,13]. *Lactobacillus* and *Bifidobacterium* are butyrate-producing bacteria [5]. This could explain, in our study, the increase in microbial species after 72h of colonic fermentation of the PS-WRB digesta, which translates into an increase in butyrate in the DC and consequently an increase in total SCFA. It should be noted that it has been reported that 10 g of dietary fiber could lead to the production of SCFA of around 100 mM [5]. This would agree with our results, where 84 mM total SCFA are obtained in the DC after 72h of fermentation of rye bread containing 15.3 g fiber/100 g bread. In addition, in a previous study of our research group, the colonic fermentation of a PS-ingredient (source of free microcrystalline PS) was evaluated by a dynamic fermentation system [14]. PS ingredient led to modifications of the microbiota composition (increase of some genera from the phylum *Firmicutes* such as *Catenibacterium* and *Coprococcus*) and of SCFA (including butyrate). It is therefore possible that the PS present in our matrix may also contribute to the butyrate generation mentioned above.

The decrease in ammonium ion could be explained by the amount of protein present in the PS-WRB (5.3 g/100 g bread). The low amount of protein compared to fiber (2.5-fold higher) could also have an inhibitory effect on proteolytic fermentation, as well as the production of SCFA that inhibits the proteolytic capacity of the enzymes. In addition, enhanced bacterial growth and carbohydrate fermentation can reduce ammonia concentrations in the gut due to a greater incorporation of nitrogen into microbial cells [15].

5. Conclusions

Our results showed that the consumption of PS-WRB could influence the growth of beneficial microbial species as *Lactobacillus* and *Bifidobacterium*, which in turn promotes the production of butyrate, a crucial energy substrate for enterocytes. This combined effect boosts the capacity to promote health while also increasing microbial diversity.

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