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Can Supplemented Skim Milk (SKM) Boost Your Gut Health?

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Abstract: The incorporation of functional ingredients, such as prebiotics and probiotics in food matrices, became a common practice in the human diet to improve the nutritional value of the food product itself. Worldwide, skim milk (SKM) is one of the most consumed food matrices, comprising all the essential nutrients desired for a balanced diet. Thus, the modulation of the human gut microbiota by SKM supplemented with different well-known functional ingredients was evaluated. Four well-studied prebiotics, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), mannan-oligosaccharides (MOS) and inulin, and one probiotic product, UL-250® (*Saccharomyces boulardii*) were added at 1% (*w*/*v*) to SKM and subjected to a gastrointestinal in vitro model. The impact of each combination on gut microbiota profile and their fermentation metabolites (i.e., short-chain fatty acids–SCFA) was assessed by quantitative polymerase chain reaction (qPCR) and high-performance liquid chromatography (HPLC), respectively. The addition of FOS to SKM had promising results, showing prebiotic potential by promoting the growth of *Lactobacillus*, *Bifidobacterium*, and *Clostridium* cluster IV. Moreover, the increment of SCFA levels and the decrease of total ammonia nitrogen were observed throughout colonic fermentation. Overall, these results demonstrate that the combination SKM + FOS was the most beneficial to the host's health by positively modulating the gut microbiota.

Keywords: functional ingredients; prebiotic; probiotic; skim milk; gut microbiota

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1. Introduction

Human dietary habits vary between individuals, and often change throughout one's lifetime, altering the gut microbiota composition and function [1]. Such modifications may be advantageous when there is an increase of beneficial bacteria (e.g., *Lactobacillus*) and/or production of specific organic acids with impact to the host's immune system (e.g., butyrate). On the other hand, when in dysbiosis, e.g., the predominance of commensal bacteria, such as *Escherichia coli* and *Bacteroides fragilis*, inflammation and other infections may rise and lead to gastrointestinal diseases and obesity, among other non-beneficial effects [2–4].

The knowledge of the impact of diet on health and well-being and the recognition of the role of gut microbiota led consumers to be more alert to food's additional health benefits [3,5,6]. Additionally, studies reinforce that everyone can achieve an optimal gut microbiota according to their lifestyle, which includes dietary habits [6].

The human gut microbiota populations living in the gut are diverse (e.g., bacteria, archaea and eukaryotes), abundant (from 10^{10} to 10^{12} live microorganisms per gram in the human colon) and in a close relationship with the host [7,8]. The main phyla found are Firmicutes and Bacteroidetes, followed by Proteobacteria and Actinobacteria, representing 93.5–98% of the bacteria present in gut microbiota. The most common genera are *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, *Clostridium* and *Escherichia* [9–11]. These microorganisms and their metabolism (e.g., microbial fermentation), play a key role in the host's

health and well-being, with special relevance to the host's nutrition (e.g., nutrient digestion/absorption), physiological (e.g., gut development) and immune systems (e.g., innate and acquired immune response) [12].

Functional ingredients are popular among consumers due to their enhanced organoleptic characteristics and potential health benefits [13]. Their incorporation in different food matrices has been demonstrated to exert a positive regulation and modulation of the gut microbiota, proactively preventing undesirable health-conditions [13]. Nevertheless, the utilization of functional ingredients must take into account the ingredient-food matrix interaction(s) and the potential adverse effects that may occur when incorrectly used [14]. Prebiotics and probiotics are examples of functional ingredients. Prebiotics are substrates, selectively utilized by the host microorganisms, conferring health benefits, while probiotics are live microorganisms that, when administrated in an adequate amount, present a health benefit to the host [15]. Probiotics are normally delivered to humans through suitable carrier food matrices, while prebiotics are incorporated in such matrices to increase nutritional and functional characteristics, while simultaneously being sensorily desirable [16,17].

Dairy products are still among the most common food matrices used to deliver probiotics and prebiotics [18–20], despite consumer demand for non-animal and/or nondairy derived functional products [21], mainly because milk is a source of high-quality protein and bioavailable amino acids, vitamins, and minerals (e.g., calcium) [22]. However, the interactions between the food matrix and the functional ingredients must be evaluated, as these interactions can define the outcome of the supplemented food. The impact of these combinations is a decisive factor when it comes to select the aptness of a functional ingredient for incorporation in a specific food matrix and, consequently, maximize its potential when fermented by the gut microbiota [15,23]. This offers the food industry the opportunity to claim additional health benefits of their supplemented products based on experimental evidence.

The supplementation of food matrices with prebiotic and probiotic ingredients is commonly tested to evaluate the effect of these ingredients on the growth of gut microbiota beneficial bacteria such as *Bifidobacterium* and *Lactobacillus.* These bacteria and their produced metabolites (e.g., short-chain fatty acids (SCFA)) can be used as health indicators and as a reference for the development of personalized nutrition strategies [24]. Such assessments can be carried out using in vitro gastrointestinal (GIT) simulation models. These models are considered the most suitable first-stage strategy to screen the potential of bioactive compounds to modulate gut microbiota and are usually less time-consuming and laborious, raising little or no ethical problems in comparison with in vivo studies [15,25,26]. However, most of the studies only assess the colonic fermentation stage, neglecting all the previous digestive and absorptive steps, which greatly contribute to the accessibility of the ingested matrix.

The aim of this study was to evaluate the impact of skim milk, supplemented with different functional ingredients, on gut microbiota modulation. Skim milk (SKM) was chosen as a food matrix not only due to its complexity as a food structure, but also because it is a nutritionally complete food on its own [22], and is widely consumed and generally well accepted. This evaluation was performed using a complete gastrointestinal model including gastric and intestinal digestion, intestinal absorption, and colonic fermentation stages.

2. Materials and Methods

2.1. Reagents/Chemicals and Apparatus

The description of all reagents/chemicals and equipment used in this study are described in Appendix A.

2.2. Preparation of Fecal Inoculum

Fresh human fecal samples from five healthy adult volunteers were obtained within the premises of the Alchemy project (Universidade Católica Portuguesa, Escola Superior de

Biotecnologia, Porto, Portugal), according to the internal established protocol, previously validated by the Health Ethics Committee from Universidade Católica Portuguesa.

Fecal samples were collected in a clean tamper-proof specimen 1 L container. The containers with the feces were placed in the OxoidTM AnaeroJar™ 2.5 L with an Oxoid™ AnaeroGen™ 2.5 L sachet, closed until opened inside of the anaerobic cabinet, a Whitley A35 workstation, and used within 2 h of collection. Under the anaerobic cabinet atmosphere (nitrogen 80%, carbon dioxide 10%, hydrogen 10%), the fecal content of each donor was sampled in equal amounts (~12 g), placed into an empty pre-weight tamper-proof specimen 1 L container, and weighed to obtain a pooled fecal inoculum to have a uniform and representative inoculum. The fecal content was diluted at 10% (*w*/*w*) in a 0.1 M phosphate-buffered saline pH 7.3 (PBS) solution with 30% (*v*/*v*) glycerol and homogenized, first manually, and further mechanically, with a Mixwel® laboratory blender for 2 min at 460 paddles beats/min [27]. Several aliquots of the pooled fecal inoculum were stored at −20 °C to avoid repeated freezing and thawing cycles.

2.3. Information Regarding the Fecal Donors

All subjects signed an informed consent form and voluntarily answered a questionnaire regarding their nutritional and lifestyle habits before their participation in the study, in accordance with the Declaration of Helsinki conduct (see Supplementary Material File S1) and to the collecting and processing of personal data guidelines, as established by Regulation 2016/679 of the European Parliament and the Council (EU) (see Supplementary Material File S2).

The volunteers had a normal omnivorous diet, without any intestinal disorders, had not ingested antibiotics or other medicines known to affect the microbiota for at least 12 months prior to the study, and were not regular prebiotics or probiotics consumers. In addition, their last hospital admission took place more than 12 months before this study. Volunteers were two males and three females aged 33.4 ± 4.92 years, with a body mass index (BMI) of 25.36 ± 2.41 kg/m^2. Detailed information on the volunteers participating in this study is displayed in Table 1.

Table 1. Volunteer Detailed Information.

Donors Information							
Donor	**Genre**	**Height (m)**	**Weight (kg)**	**Age (years)**	**BMI (kg/m^2)**	**Dairy Consumption**	**Regular Exercise**
D1	Female	1.76	81	35	26.15	Yes	Yes
D2	Male	1.82	97	25	29.37	Yes	Yes
D3	Female	1.62	60	40	22.86	Yes	Yes
D4	Male	1.68	72	32	25.51	Yes	Yes
D5	Female	1.55	55	35	22.89	Yes	Yes

2.4. Human In Vitro Gastrointestinal Tract (GIT) Simulation Model

An in vitro GIT simulation model adapted from Brodkorb et al. (2019) [28] (INFOGEST protocol) with modifications introduced by Carvalho et al. (2021) [27] (in vitro human colonic fermentation protocol) was carried out. This protocol is somehow similar to other fermentation protocols previously published, however with modifications regarding e.g., basal media, percentage of inoculum and pH control during fermentation. In a first stage, SKM supplemented with galacto-oligosaccharide (GOS), mannan-oligosaccharides (MOS), fructo-oligosaccharides (FOS), inulin and *Saccharomyces boulardii* (SB) (1% (*w*/*v*)) was submitted to gastric and intestinal digestion, followed by intestinal absorption, using a 1 kDa dialysis membrane. For the colonic fermentation, the frozen pooled fecal inoculums prepared previously were used and seven conditions (duplicated) were tested: (I) inoculum control (IC), with fecal slurry only; (II) digested SKM; (III) to (VI) digested SKM supplemented with 1% (*w*/*v*) FOS, GOS, MOS, inulin and SB respectively. The SKM used had a nutritional composition of 54.1% carbohydrates, 34.5% protein, 1% lipids and 0% fibers according to the available nutritional information of the product.

Sterile stirred batch culture fermentation vessels of 300 mL were set up and aseptically filled with 135 mL sterile basal nutrient medium according to de Carvalho et al. (2019) [29] and gassed overnight with O_2-free N_2 and with continuous agitation. Each condition was assessed in duplicate, and digested substrates were added at 1% (*w*/*v*) aseptically (by flaming the entry/sampling port). Once the substrates were properly mixed with the basal media, each vessel was inoculated with 15 mL fecal inoculum (these inoculums were previously submitted to a two-glycerol wash-out cycles according to de Carvalho et al. (2021) [27] to eliminate the glycerol, which was used as a cryopreservative during storage.

A FerMac 260 pH controller was used to maintain the pH in each vessel between 6.7 and 6.9 (the pH of the human distal colon) (Sánchez-Patán et al., 2012) [30], and the temperature was kept at 37 °C with the help of a water bath. Batch fermentations were run for 48 h and samples (10 mL) were taken aseptically from each vessel at 0, 6, 24, 30, and 48 h for short chain fatty acids (SCFA) and lactate analysis done by HPLC, ammonium (NH_4^+) concentration measurement, using an ion-selective electrode 9663 of ammonium and bacterial enumeration by real-time quantitative polymerase chain reaction (qPCR). Immediately after collection, samples were placed in ice to stop the fermentation, centrifuged at 4 °C, 4696× *g* for 5 min, and the supernatant was collected for HPLC analysis. The pellet was resuspended in 10 mL of a 0.1 M PBS solution, vortexed, and centrifuged (as described previously). The supernatant was discarded, and the washing cycle repeated one more time. Pellets from 0, 24 and 48 h were stored at −20 °C until qPCR analysis.

2.5. Bacterial Enumeration by Real-Time qPCR

The total DNA was extracted from the stored pellets using an innuPREP Stool DNA Kit-IPC16 and an automated nucleic acids extractor following the manufacturer's instructions (Analytik-Jena, Jena, Germany). The quantification of DNA concentration was measured by a Scandrop, and the final DNA concentration of each sample adjusted to 20 ng/µL.

The targeted groups, primer sequences, amplicon sizes and literature references are depicted in Table 2. Conditions for qPCR reactions were prepared to a final volume of 10 µL, containing of 1 × NZYSpeedy qPCR Green Master Mix, 1 µM of each primer (forward and reverse), 2 µL of DNase/RNase-free water and 1 µL of template DNA. In the negative control, 1 µL of DNase/RNase-free water was used instead of template DNA. The cycling conditions were 95 °C for 10 min (polymerase activation), 95 °C for 10 min (denaturation), then 45 cycles of 63 °C for 1 min (annealing), and extension at 72 °C for 15 min. Samples were assessed in triplicate. Additionally, an analysis of the melting curve was performed. For the bacterial quantification, the DNA of bacterial monocultures were used to create standard calibration curves (Tables 2 and 3). Briefly, for each set of primers, five decimal dilutions of bacterial DNA were prepared to plot a standard calibration curve, which correlates the cycle threshold (Ct) values and log colony-forming unit (CFU) per mL.

Table 2. Bacteria monocultures used as genomic DNA standard for calibration curves and group-specific primers based on 16S rDNA sequences to profile colonic samples.

Primer	Target Group or Organism	Genomic DNA Standard	Sequence (5′-3′)	Amplicon Size (bp)	Reference
Firm	Firmicutes	*Lactobacillus gasseri* DSM 20077	**F:** ATGTGGTTTAATTCGAAGCA **R:** AGCTGACGACAACCATGCAC	126	[31]
Lac	*Lactobacillus* group		**F:** CACCGCTACACATGGAG **R:** AGCAGTAGGGAATCTTCCA	341	[32,33]
Bdt	Bacteroidetes	*Bacteroides intestinalis* DSM 17393	**F:** CATGTGGTTTAATTCGATGAT **R:** AGCTGACGACAACCATGCAG	126	[31]
Bac	*Bacteroides*		**F:** ATAGCCTTTCGAAAGRAAGAT **R:** CCAGTATCAACTGCAATTTTA	501	[34]
Bif	*Bifidobacterium*	*Bifidobacterium animalis* ssp. *lactis BB-12* DSM 15954	F: CGCGTCYGGTGTGAAAG **R:** CCCCACATCCAGCATCCA	244	[35]
Enb	*Enterobacteriaceae* family	*Salmonella enteritidis* subsp. *enterica* ATCC 13076	**F:** CATTGACGTTACCCGCAGAAGAAGC **R:** CTCTACGAGACTCAAGCTTGC	195	[36]
CIV	*Clostridium* cluster IV	*Clostridium leptum* DSM 753	**F:** TTACTGGGTGTAAAGGG R: TAGAGTGCTCTTGCGTA	580	[37]

F—forward; R—reverse.

Table 3. Bacteria monocultures used as genomic DNA standard for calibration curves and their growth conditions.

Bacteria Monoculture	Media Broth	Media Agar	Incubation Conditions
Lactobacillus gasseri DSM 20077	MRSB + 0.1% (*w*/*v*) cysteine	MRSA+ 0.1% (*w*/*v*) cysteine	Anaerobic 37 °C 2 days
Bacteroides intestinalis DSM 17393	TSB+ 5% DSB	CBA + 5% (*v*/*v*) DSB	
Bifidobacterium animalis ssp. *lactis BB-12* DSM 15954	MRSB + 0.1% (*w*/*v*) cysteine	MRSA + 0.1% (*w*/*v*) cysteine	
Salmonella enteritidis subsp. *enterica* ATCC 13076	MHB	MCA	
Clostridium leptum DSM 753	PYMB	PYMA	

CBA—Columbia base agar; DSB—defibrinated sheep blood; F—forward; MCA—MacConkey agar; MHB—Mueller-Hinton broth; MRSB/A—de man, de rogosa and sharpe broth/agar; R—reverse; PYMA/B—peptone yeast maltose broth/agar; TSB—tryptic soy broth.

2.6. Determination of Organic Acids Produced Throughout Fermentation

The supernatants collected after centrifugation were filtered (0.22 µm) and directly analyzed by HPLC in duplicates, as described in de Carvalho et al. (2021) [27], with slight modifications. Conditions for the HPLC system consisted in an Agilent 1260 II series HPLC instrument with a refractive index (RI) detector and diode array detector (DAD) at 220 nm, and an ion-exclusion Aminex HPX-87H column operated at 50 °C. A 5 mM sulfuric acid (H_2SO_4) mobile phase was used at a flow rate of 0.6 mL/min with a running time of 40 min and injection volume of 10 µL. Lactate, acetate, propionate, butyrate, isobutyrate and isovalerate were identified and quantified using their corresponding calibration curves (2 mM to 80 mM).

2.7. Measurement of Total Ammonia Nitrogen Concentration

An ion-selective electrode 9663, at constant temperature (room temperature, 20 °C) and pH (6.7–6.9), was used for the measurement of ammonium concentration. According to the manufacturer's instructions, 300 µL of 1M $MgSO_4$ (ionic strength adjuster) was added to 3 mL of the supernatants collected after centrifugation, and readings were carried out in duplicate using an electrode. For quantification, a standard calibration curve of NH_4Cl (2 mM to 55 mM) was used. Total ammonia nitrogen concentration was calculated according to the equation below [38]:

$$\frac{\left[NH_4^+\right]}{\left[NH_3 + NH_4^+\right]} = 1 - \frac{1}{1 + 10^{pKa - pH}}$$

where, (NH_4^+) is the ammonium ion concentration, (NH_3 + NH_4^+) is the total ammonia nitrogen concentration, and *pKa* is the acid dissociation constant that can be expressed as a function of temperature (*T*) using the following equation [38]:

$$pKa = 4 \times 10^{-8} T^3 + 9 \times 10^{-5} T^2 - 0.0356T + 10.072$$

2.8. Statistical Analysis

Statistical analysis was carried out using IBM SPSS Statistics 27 software (IBM, Chicago, IL, USA). The data's normality of each condition for each bacterial group, organic acid and total ammonia nitrogen concentration at the same sampling time was evaluated using the Shapiro–Wilk's test. As the samples followed normal distribution, means were compared considering a 95% confidence interval, using a one-way ANOVA coupled with Tukey's post-hoc test.

3. Results

3.1. Bacterial Profile on the Fermentations

Figure 1A–G, shows the bacterial detection and quantification of different bacterial populations, by qPCR at fermentation at times of 0, 24, and 48 h.

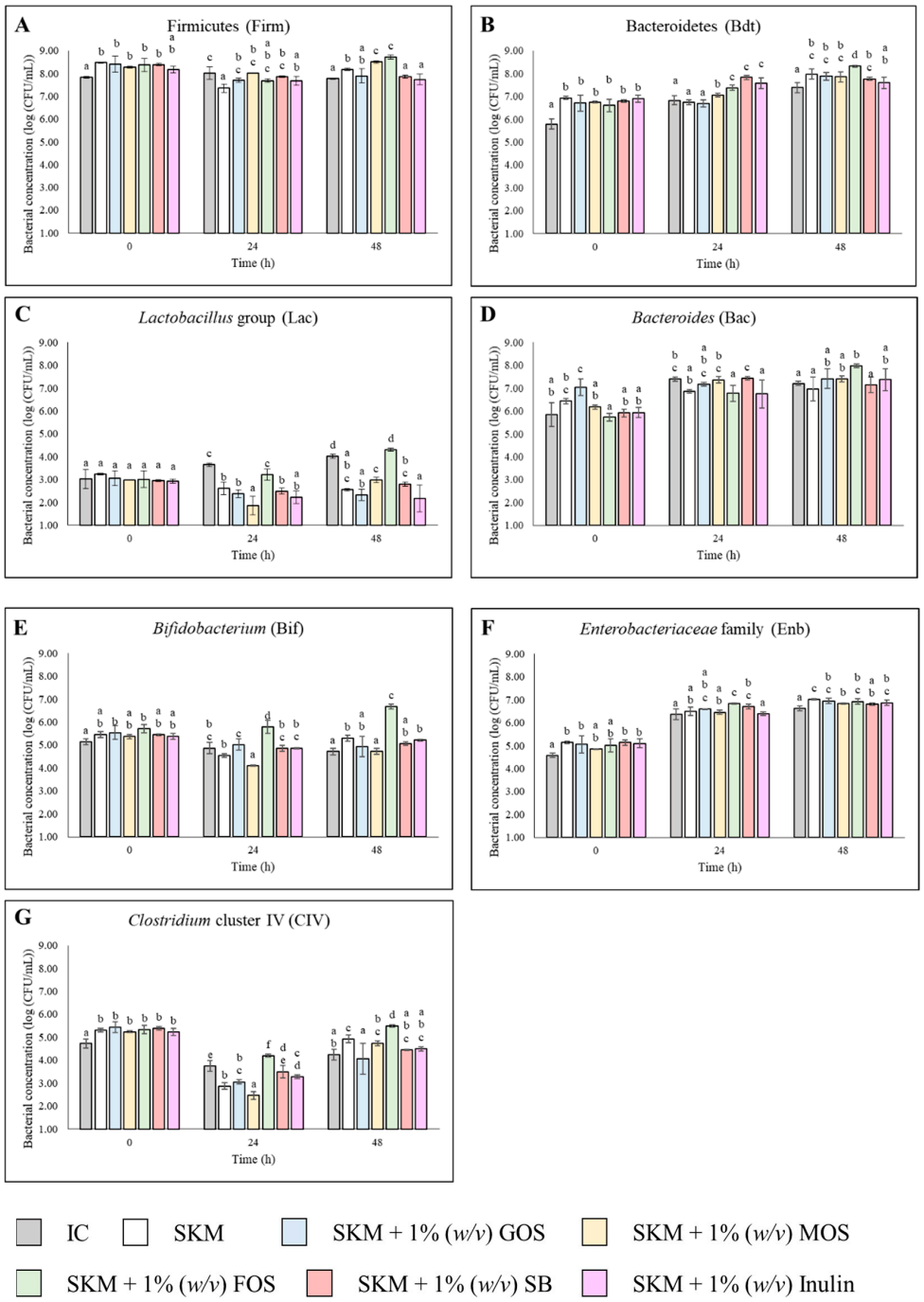

Figure 1. (**A**–**G**)—Bacterial quantification (log (CFU/mL), mean ± SD) of the different bacterial populations present in the fecal fermentations for the different condition. (**A**) Firmicutes (Firm), (**B**) Bacteroidetes (Bdt), (**C**) *Lactobacillus* group (Lac), (**D**) *Bacteroides* (Bac), (**E**) *Bifidobacterium* (Bif), (**F**) *Enterobacteriaceae* family (Enb) and (**G**) *Clostridium* cluster IV (CIV). Different letters mark statistically significant ($p < 0.05$) differences between each condition for each bacterial group at the same sampling time. IC—inoculum control; SKM—skim milk; GOS—galacto-oligosaccharide; MOS—mannan-oligosaccharides; FOS—fructo-oligosaccharides; SB—*S. boulardii*.

At 0 h, all conditions had the same bacterial cell concentration ($p < 0.05$), except the IC condition, for Firmicutes (Firm), Bacteroidetes (Bdt), *Bifidobacterium* (Bif), *Enterobacteriaceae* family (Enb) and *Clostridium* cluster IV (CIV) and SKM + GOS for *Bacteroides* (Bac) ($p > 0.05$). The highest bacterial DNA amount quantified was Firm (7.8–8.5 log (CFU/mL)) followed by Bdt (5.8–6.9 log (CFU/mL)), Bac (5.7–7.0 log (CFU/mL)), CIV (4.7–5.4 log (CFU/mL)), Bif (5.2–5.7 log (CFU/mL)), Enb (4.6–5.2 log (CFU/mL)) and *Lactobacillus* group (Lac) with the lowest quantity (2.9–3.2 log (CFU/mL)) (Figure 1 and Table S1).

At 24 h of colonic fermentation, as shown in Figure 1 and Table S1, the bacterial concentration of Firm, Bac and Enb, was higher for IC (8.1 log (CFU/mL)), SKM + SB (7.4 log (CFU/mL)) and SKM + FOS (6.8 log (CFU/mL)), respectively. However, these conditions were not statistically different from the remaining conditions at the same time point ($p > 0.05$). The supplementation of SKM with FOS, SB and inulin had the highest bacterial concentration of Bdt at 24 h, i.e., 7.4, 7.8 and 7.6 log (CFU/mL), respectively, and were statistically different from the other conditions tested ($p < 0.05$). Supplementation with FOS originated higher values of Bif (5.8 log (CFU/mL)) and CIV (4.2 log (CFU/mL)), which were statistically different when compared with the remaining conditions ($p < 0.05$). The highest concentration of Lac was found for IC condition (3.6 log (CFU/mL)) and SKM + FOS (3.2 log (CFU/mL)) ($p < 0.05$). On the other hand, SKM + MOS presented significantly lower ($p > 0.05$) concentrations of Bif (4.1 log (CFU/mL)) and CIV (2.5 log (CFU/mL)) when compared with the remaining conditions.

At 48 h, SKM + FOS was the condition with the highest concentration of Firm (8.7 log (CFU/mL)), Bdt (8.3 log (CFU/mL)), Bif (6.7 log (CFU/mL)) and CIV (5.5 log (CFU/mL)) and statistically different from the remaining conditions at the same time point for these same bacterial groups ($p < 0.05$) (Figure 1 and Table S1). The supplementation with FOS was also the combination which resulted in a higher quantity of Bac (8.0 log (CFU/mL)) and Lac (4.3 log (CFU/mL)); however, it was not statistically different from the other conditions at the same time point ($p > 0.05$). The condition with the highest quantity of bacteria from Enb was SKM (i.e., 7.0 log (CFU/mL)); however, it was not statistically different ($p > 0.05$) from SKM + GOS and FOS, both with approximately 7 log (CFU/mL). From all tested conditions, the one with the lowest amount of all targeted bacteria, except for Lac, was IC, although not statistically significant ($p > 0.05$).

These results have shown that the biodiversity of the fecal material changed during fermentation (greater bacteria quantity present at 48 h) and that the tested substrates had a distinct impact throughout the fermentation course (Figure 1A–G and Table S1).

3.2. Colonic fermentations SCFA Profile

Figure 2A–F shows the concentration of lactate, acetate, propionate, butyrate, valerate and isovalerate produced throughout the 48 h of fecal fermentations.

The conditions which produced the highest levels of SCFA (i.e., acetate + propionate + butyrate) at the end of the 48 h fermentation were FOS (93 mM) > SKM (74 mM) > SB (72 mM) > inulin (68 mM) > GOS (65 mM) > MOS (59 mM) > IC (25 mM) (Figure 2 and Table S2). This trend of SCFA production between the different conditions was always verified at all sampling times, with the exceptions of 6 and 24 h (Table S2).

Lactate was the organic acid with a different production pattern regarding the other organic acids. All conditions tested produced lactate; however, it disappeared between 24 and 30 h of the fermentation process. Lactate was not detected at 24 h for the IC and SKM + FOS conditions, whereas it only disappeared at 30 h of fermentation for the remaining conditions. The highest lactate production was found for SKM + FOS at 6 h (i.e., 25.7 mM), while IC was the condition with the lowest lactate production at the same time point (i.e., 0.08 mM). These conditions were statistically different from the remaining conditions ($p < 0.05$) (Figure 2A and Table S2).

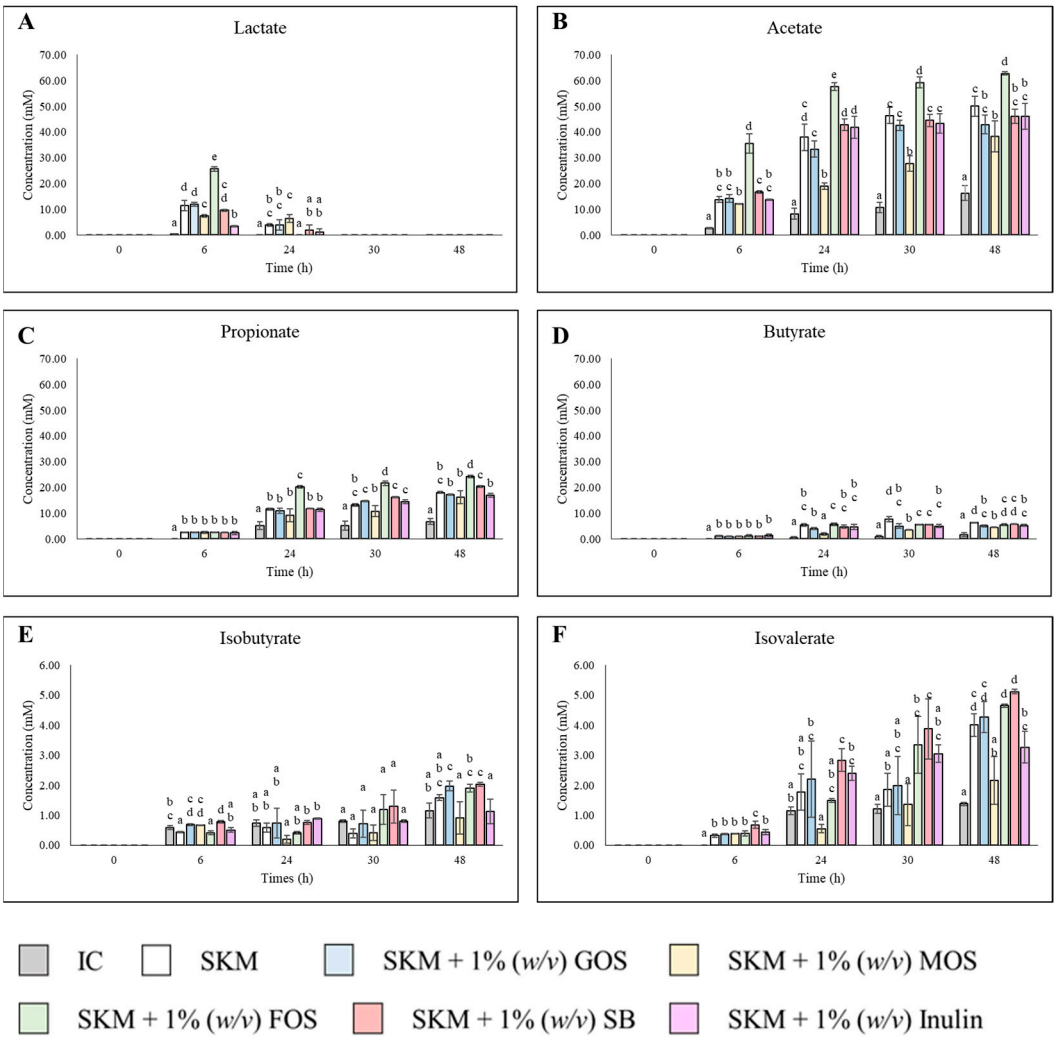

Figure 2. (**A**–**F**)—Concentration (mM, means ± SD) of the different organic acids produced during 48 h of fecal fermentation: (**A**) lactate, (**B**) acetate, (**C**) propionate, (**D**) butyrate, (**E**) isobutyrate and (**F**) isovalerate. Different letters mark statistically significant ($p < 0.05$) differences between each condition at the same sampling time. IC—inoculum control; SKM—skim milk; GOS—galacto-oligosaccharide; MOS—mannan-oligosaccharides; FOS—fructo-oligosaccharides; SB—*S. boulardii*.

The highest production of acetate, at all-time points, was found for SKM + FOS (i.e., 6 h-35.5 mM, 24 h-57.8 mM, 30 h-59.3 mM and 48 h-62.7 mM), and it was statistically different from the other conditions ($p < 0.05$). The lowest acetate production and statistically different from the other conditions ($p < 0.05$) was IC (i.e., 6 h-2.6 mM, 24 h-8.3 mM, 30 h-10.7 mM and 48 h-16.3 mM) (Figure 2B and Table S2).

Regarding the production of propionate, at 6 h, no statistical differences between the conditions supplemented with the functional ingredients tested and the SKM alone was observed. After 24 h of fermentation, SKM + FOS became the condition with the highest values of propionate ($p < 0.05$) until the end of the fermentation process (i.e., 24 h-20.3 mM, 30 h-21.6 mM and 48 h-24.3 mM). Contrarily, IC had the lowest propionate concentration ($p < 0.05$) at all time points (i.e., 6 h–non detectable, 24 h-5.1 mM, 30 h-5.2 mM and 48 h-6.7 mM) (Figure 2C and Table S2).

As shown in Figure 2D–F and Table S2, throughout the 48-h fermentation, none of the tested ingredients increased the concentration of butyrate, isobutyrate and isovalerate in comparison with the SKM condition. The concentration of these acids remained similar ($p > 0.05$) among all conditions tested, except for MOS, which had lower productions of

butyrate (i.e., 48 h-4.6 mM) and isovalerate (i.e., 48 h-0.9 mM) when compared with SKM only (i.e., 48 h-6.4 mM butyrate and 1.6 mM isobutyrate) ($p < 0.05$).

Regarding the propionate: acetate ratio (Table S2), all conditions that included SKM remained similar at all-time points (i.e., 6 h-0.1–0.2, 24 h-0.3–0.5, 30 h-0.3–0.4 and 48 h-0.4). However, at 6 h, no propionate was detected for the IC condition, thus propionate: acetate ratio was only considered from 24 h onwards, when propionate values were detectable and followed the same pattern as the remaining conditions (i.e., 24 h-0.6, 30 h-0.5 and 48 h-0.4).

3.3. Colonic Fermentations Total Ammonia Nitrogen Profile

Figure 3 shows the total ammonia nitrogen produced throughout the 48 h of fecal fermentations.

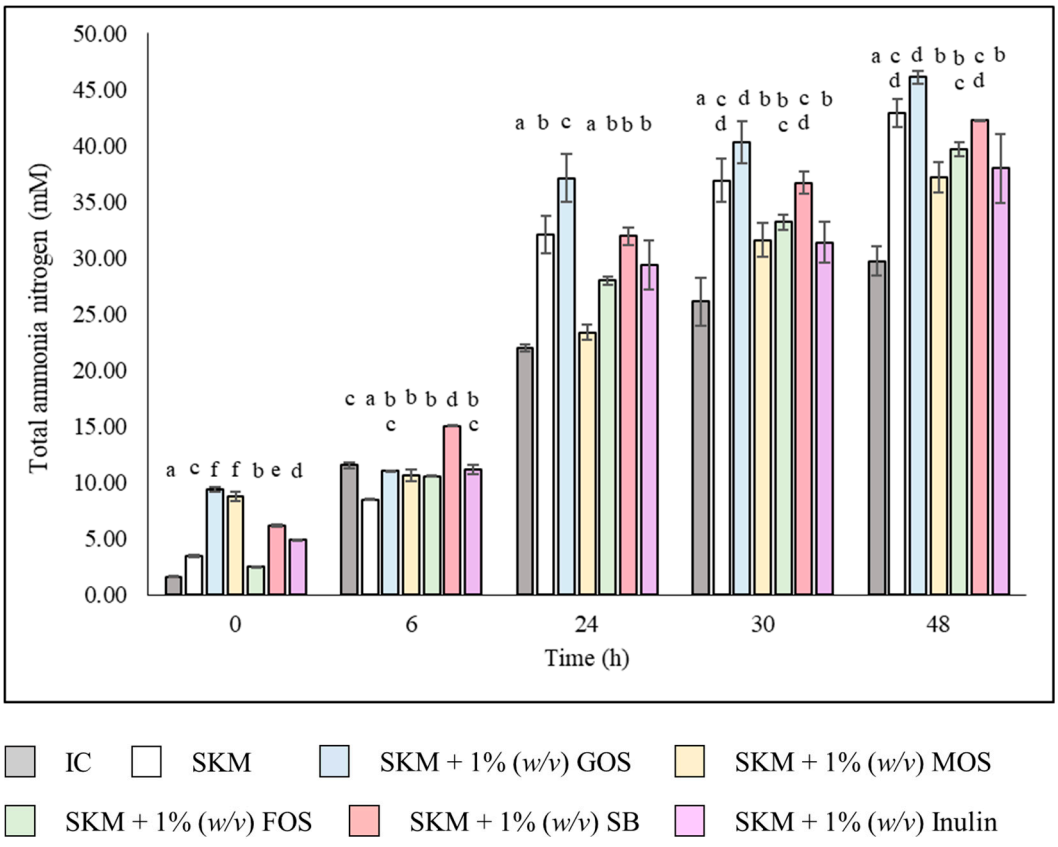

Figure 3. Concentration (mM, means ± SD) of total ammonia nitrogen produced during 48 h of fecal fermentation. Different letters mark statistically significant ($p < 0.05$) differences between each condition at same sampling time. IC—inoculum control; SKM—skim milk; GOS—galacto-oligosaccharide; MOS—mannan-oligosaccharides; FOS—fructo-oligosaccharides; SB—*S. boulardii*.

At 0 h, the total ammonia nitrogen concentration was different in all conditions ($p < 0.05$), except for the combinations SKM + GOS and SKM + MOS (i.e., 9.3 mM and 8.6 mM respectively) ($p > 0.05$) (Figure 3 and Table S3).

At 6 h, the condition with the lowest production of total ammonia nitrogen was SKM (i.e., 8.5 mM), whereas SKM + SB had the highest value (i.e., 15 mM) ($p < 0.05$) (Figure 3 and Table S3).

The conditions with the lowest concentration of total ammonia nitrogen at 24 h were IC (i.e., 21.3 mM) and SKM + MOS (i.e., 23.1 mM) ($p < 0.05$). On the other hand, SKM + GOS was the condition with the highest production of total ammonia nitrogen, from 24 h until 48 h (i.e., 24 h-36.4, 30 h-39.6 mM and 48 h-45.8 mM) ($p < 0.05$). At 30 and 48 h, IC was, once again, the condition with lower production of total ammonia nitrogen (i.e., 25.4 mM and 29.3 mM, respectively) (Figure 3 and Table S3).

From the tested functional ingredients, at 48 h, SKM + MOS, + FOS and +inulin, were the conditions with the lowest concentrations of total ammonia nitrogen, i.e., 36.7 mM, 39.4 mM, 37 mM, respectively ($p < 0.05$).

4. Discussion

The purpose of this work was to evaluate if the addition of different well-acknowledged and studied functional ingredients to SKM would add any health benefits to the SKM itself by, for example, promoting the growth of beneficial gut bacteria or maintaining/inhibiting the growth of the undesirable bacterial groups. It was also to establish which combination would be the most effective in positively modulating the gut microbiota. The impact of supplemented SKM on gut microbiota modulation (i.e., bacteria growth, organic acid, and total ammonia nitrogen production) was assessed after subjecting the food sample to digestion and absorption protocols. The unabsorbed subtract was further submitted to an in vitro batch fermentation model using a pooled human fecal inoculum. The utilization of pooled fecal samples is currently a common practice, as it guarantees a better representation of the bacterial communities present in the lumen of the colon [3,39–41].

The non-absorbable (indigestible) carbohydrates (polysaccharides and oligosaccharides), proteins/peptides and lipids, which become the substrate for the beneficial bacteria of the gut microbiota when arriving to the colon, are considered prebiotics [42]. However, the most studied prebiotic with well documented benefits in both humans and animals are the indigestible carbohydrates, namely FOS, GOS and inulin [17,42,43], whereas MOS is still considered an emerging prebiotic [43]. The functional ingredients used in this study are the well-known and extensively studied pre-and probiotics [17,43,44]. Plant-based FOS and inulin, from chicory root, animal driven GOS, from cow's milk and MOS, from the yeast cell wall, were the prebiotics tested. *Saccharomyces boulardii*, a non-pathogenic yeast strain commercialized worldwide and commonly used as a probiotic for humans to prevent and treat diarrhea and intestinal disfunctions, such as irritable bowel syndrome (IBS) and Crohn's disease [44], was the selected probiotic.

The impact of SKM supplemented with different functional ingredients on bacterial biodiversity throughout colonic fermentation was assessed by qPCR, and focused on seven important bacterial groups present in the human intestinal microbiota: Firm and Bdt phylum, Enb, CIV, Lac, Bac and Bif. These bacterial groups were selected due to their relevance for human health and their role in colonic fermentation [45,46]. The use of a molecular method, such as qPCR, is a useful tool to overcome the limitations of culture-dependent methods, enabling a more precise description of the microbiota composition [47].

Figure 1 (and Table S1) shows that, at 0 h of fermentation, the fecal inoculum, had higher concentrations of bacteria belonging to the Firm phylum (i.e., 7.8–8.5 log (CFU/mL)) than to the Bdt phylum (i.e., 5.8–6.9 log (CFU/mL)), as previously verified in other studies [47–49]. Firm and Bdt are the two most predominant phyla, representing 90% of the bacterial cells, present in a healthy human adult gut microbiota [12,50]. The relative proportions of these Firm and Bdt in the gut microbiota can vary and are influenced by a range of factors (e.g., host genetics, dietary habits, age, lifestyle) [51,52]. Bacteria from the Firm phylum are positively related with the degradation of polysaccharides and butyrate production, and are more effective in extracting energy from food. These bacteria are associated with weight gain, while Bdt are related to the degradation of carbohydrates and propionate production [12,53,54]. Additionally, Figure 1 and Table S1 show that Bac is the most predominant bacterial group, followed by CIV, Bif, Enb and Lac. These results are in accordance other published studies which state that in adult human fecal microbiota, Bac, CIV and Bif are the most dominant groups of bacteria, with Bac being the most prevalent group, while Enb (specially *Escherichia*) and Lac exist in lower concentrations (sub-dominant genera) [11,39,55–58]. Its worthy of note that statistically significant differences ($p < 0.05$) were found in some bacterial groups (i.e., Firm, Bdt, Bac, Bif, Enb and CIV) at the beginning of the fermentation. This is most likely related to the intrinsic differences associated with the biological samples, such as the fecal material. A pooled fecal inoculum

from five donors was used as the fermentation inoculum, a common procedure to reduce the inter-variability of the inoculums [59].

Dairy products and milk itself have been associated with gut microbiota health promotion [60–62]. However, when supplemented with FOS, a significant growth of beneficial bacteria, namely Lac, Bif, and CIV (24 and 48 h) and Bac (48 h) was observed, in comparison with SKM only ($p < 0.05$) (Figure 1 and Table S1). These results are somehow expected, as their beneficial properties and role in human nutrition, health and energy metabolism of FOS are currently well-established [43,63,64]. The most acknowledged nutritional effect of FOS is the selective stimulation of Bif and Lac growth/activity and the prevention of harmful bacteria colonization such as *E. coli* and *Salmonella typhimurium* in the human colon [17,41,63,65]. In the present study, SKM + FOS promoted the growth of Lac, Bif, and CIV groups; however, it did not inhibit the growth of Enb, as reported previously in several animal and human studies compiled by Azad et al. (2020) [43]. This result is most likely related with the combination used, which offers different nutrients and conditions for bacteria proliferation than it does for FOS alone.

Bacterial growth throughout colonic fermentations consequently increases the production of metabolites such as SCFA and branched-chain fatty acids (BCFA). The main SCFA are acetate, propionate, and butyrate, which together represent 90–95% of the total SCFA, and at a smaller concentration (~5% of total SCFA) are the BCFA, such as isobutyrate and isovalerate [66,67]. The production of SCFA improves the gut health through the promotion of the microbial growth and microbial community structure (by balancing the interactive competition between "beneficial" and "harmful" bacteria), the secretion of bacteriocin, and the enhancement of the immune barrier [64,66–70]. These SCFA are also important as physiological signaling molecules, adjusting biological processes related with the host's health and diet, playing a key-role in regulating responses of the gut microbiota fermentation to the host's metabolism [64]. Additionally, some SCFA ratios are associated with specific metabolic disorders. For example, the propionate: acetate ratio is often used to screen an ingredient's potential lowering cholesterol capacity when subjected to colonic fermentation. This association is due to the role of acetate in cholesterol and triacylglycerols synthesis and of propionate in the inhibition of lipogenic enzyme activity. Thus, the greater the ratio the greater the inhibition of cholesterol [41,71]. According to different studies, the addition of prebiotic oligosaccharides may promote the growth of probiotics and symbiotics, leading to an increase in SCFA production [41,72–76].Other important metabolites, namely lactate, are also produced throughout colonic fermentations [77,78].

As anticipated, the condition with the lowest production of organic acids was IC (Figure 2 and Table S2), as there are no additional nutrients to feed the fecal bacteria and promote the fermentation course [27,79–82]. The combination SKM + FOS originated the highest concentration of SCFA in comparison with SKM only. These results are in accordance with other studies previously published and also corroborate the concentrations found in this study, in which SKM + FOS, at 48 h, showed the higher growth of bacteria (i.e., Bac, Lac, Bif, and CIV) directly related to the production of acetate, propionate and butyrate [46,67,70]. The addition of FOS significantly increased ($p < 0.05$) the production of acetate and propionate at 24 h when compared with the remaining conditions. *Bacteroides*, *Lactobacillus*, *Bifidobacterium* and *Clostridium* genera are well-known for their role on the production of acetate and propionate [46].

The type of substrate present during the colonic fermentations will have a direct impact on the gut bacterial population's growth and metabolism, which are dominantly saccharolytic and/or proteolytic [83,84]. In this study, most of the nutrients provided as substrate are carbohydrates and proteins, therefore there is expected to be an increase in the bacterial metabolism. The SCFA concentration obtained with the condition IC, at 24 h and 48 h (Figure 2 and Table S2), are within the range of concentrations reported in other studies, i.e., 3–25 mM acetate, 1.5–7 mM propionate and 1.5–5 mM butyrate [27,79–82]. Moreover, at 48 h, the SKM condition presented 50, 18 and 6 mM of acetate, propionate and butyrate,

respectively, in accordance to previous experiments carried out by Carvalho et al. (2021) (i.e., 56 mM acetate, 16 mM propionate and 5 mM butyrate) [27].

The molar ratio of acetate, propionate, and butyrate, throughout the colonic fermentation, was similar among all combinations and within the values reported in previous studies, ranging from 3:1:1 to 10:2:1 [67,70,85,86]. The exceptions were: (a) SKM + FOS, which resulted in a higher concentration of acetate and propionate in comparison with butyrate at all-time points; (b) at 6 h, higher levels of acetate were detected, which changes the expected ratios and may be due to the early stage of fermentation, and (c) the IC condition for all the time points, most likely due to absence of nutrients (Table S2). Moreover, none of the supplemented functional ingredients assessed in this study showed the potential of lowering the cholesterol production according to the propionate: acetate ratio (Table S2) mentioned previously.

The main SCFA, namely acetate, propionate and butyrate, are commonly associated to the reduction of the survival of acid-sensitive bacteria, such as Enb [41]. In this study, only SKM + MOS decreased the presence of Enb in comparison with SKM ($p < 0.05$). However, this reduction is not related to the SCFA production, as the condition SKM had higher concentrations of SCFA throughout colonic fermentations. Moreover, the tested MOS (Ohly-GO® MOS) has been linked to the limitation of Enb colonization in animal studies [87].

Lactate is an organic acid usually produced by e.g., Lac and Bif in the first hours of fermentation, which disappears normally at 24 h due to its consumption by gut microorganisms, such as Bac and *Roseburia*, which converts lactate into acetate, propionate, and butyrate. This metabolic process is known as cross-feeding and limits the accumulation of lactate in the colon, preventing metabolic acidosis [29,67,70,82]. A cross-feeding phenomenon was observed in all combinations (Figure 2A). Between 6 h and 30 h, results showed lactate production, and its further consumption along with an increase of acetate, propionate, and butyrate concentrations after its depletion.

Another point worthy of highlighting regarding SCFA production is the concentration of the tested ingredient used to supplement SKM. In de Carvalho et al. (2021) [27], the supplementation of SKM with FOS at 0.1% (w/v), did not increase the fermentative potential of the gut microbiota, whereas in this study when FOS was incorporated at 1% (w/v), an increase of this fermentative potential was observed. Therefore, those results were most likely due to lower FOS concentrations used to supplement SKM.

Ammonia is a metabolite related to the protein fermentation of the bacteria in the colon, i.e., it is an end product of the proteolytic activity of the gut microbiota [51,88]. Although ammonia is a potentially toxic compound to the host, it is also used as a nitrogen source by the microbiota and mostly excreted via the stool and urine or absorbed in the gut [51,88]. According to Leschelle et al. (2002) [89], there is no evidence of a cytotoxic effect against the colon epithelium up to 50 mM NH_4Cl. Lesions on gastric mucosa were only found at ammonia concentrations above 125 mM [90].

Total ammonia nitrogen values found in all tested combinations and conditions were within the normal range and below the toxic levels. Fermentation and SCFA production promote the reduction of the luminal and fecal pH, which decreases peptide degradation, and, consequently, the formation of ammonia driven from the amino acids deamination [70,88,91]. The total ammonia nitrogen concentration levels are expected to increase over time, due to protein degradation, and it is believed that this is an indicator of protein degradation during colonic fermentation. In humans, the fecal ammonia concentration varies between 12 mM and 30 mM and increases with high protein intake diets [85]. As expected, the condition with the lowest concentration of total ammonia nitrogen at 48 h was IC ($p < 0.05$), most likely due to the absence of nutrients, as discussed previously. The addition of FOS, MOS and inulin to SKM decreased the total ammonia nitrogen concentration throughout the fermentation, with the lowest levels at 48 h ($p < 0.05$). These results are associated to the production of SCFA, the increased activity of saccharolytic bacteria, and the decreased activity of proteolytic bacteria [84,92]. Other studies have also

found that the consumption of fermentable carbohydrates (i.e., inulin, resistant starch, pectin and arabinogalactan) reduced fecal ammonia concentrations [93–95]. On the other hand, SKM only, SKM + GOS, and SKM + SB showed the highest total ammonia nitrogen production, which may be related to the nutritional similarities between SKM and GOS that allows greater proteolytic activity by the bacteria present in the fecal inoculum and to the nutritional content of SB. At 24 h, all conditions had concentrations of total ammonia nitrogen within the ranges of ammonia concentration normally found in human feces (i.e., 21–36 mM).

The mechanisms behind the modulation of gut microbiota composition by the diet is still unclear. Short-term and long-term interventions stimulate different variations in the gut microbiota [96]. According to David et al. (2014) [97], the gut microbiota has the ability to change and reshape its composition quickly by a short-term diet regimen (i.e., five consecutive days). It is important to point out that the results obtained in this study are a preliminary screening for the potential impact that SKM supplementation with functional ingredients may have on the consumer's gut microbiota and consequently on its heath. Although the in vitro approach is a well-accepted and useful tool, clinical trials (in vivo assays) are still required to fully explore the "true" benefits that food matrices (namely skim milk) supplementation could have in humans.

Overall, this study enabled the evaluation of the impact that SKM and SKM combined with different functional ingredients have on gut microbiota modulation. The extent of such modulation needs to be carefully addressed, as different combinations are likely to affect and modulate the gut population in different ways. Moreover, this study has shown the relevance of using a complete in vitro model and that the nutrients present in the digested SKM are used differently by the intestinal microbiota.

5. Conclusions

The inclusion of functional ingredients, such as prebiotics and probiotics, in a variety of food matrices has become a common practice within the food industry, mostly due to their beneficial impact on gut microbiota modulation and the consequent health boost to the host. Skim milk is a highly nutritional foodstuff and is often used as a carrier for the delivery of specific functional ingredients and/or bioactive molecules. In this study, SKM supplemented with FOS was the combination which provided the most evident additional health benefits to the host. Namely, these are the promotion of the growth of beneficial bacteria (i.e., Lac, Bif and CIV), an increase in SCFA production (i.e., acetate and propionate), and a decrease in the total ammonia nitrogen concentration during colonic fermentation. In sum, this study is relevant to food industry research because it provides valuable information on supplementing dairy products with pre- and/or probiotics and enabling additional health and nutritional benefit claims. However, further in vivo assays are required to fully validate in vitro results.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8030126/s1, File S1. Informed consent form. File S2. Consent to the collecting and processing of personal data. Table S1. Bacterial quantification (log (CFU/mL), mean ± SD) of the different bacterial populations present in the colonic fermentations for the different conditions. Table S2. Concentration (mM, means ± SD) of the different organic acids produced during 48 h of colonic fermentation. Table S3. Concentration (mM, means ± SD) of total ammonia nitrogen produced during 48 h of colonic fermentation.

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Institutional Review Board Statement: Ethical review and approval were waived for this study, as it was conducted according to the internal rules legally established, based on the research ethics recommendation and approved by the Health Ethics Committee, from Universidade Católica Portuguesa.

Informed Consent Statement: All subjects involved in this study signed an informed consent form and permission to collect and process their personal data (anonymously), within the meaning of Regulation 2016/679 of the European Parliament and the Council (EU) (see Supplementary Material Table S3).

Data Availability Statement: The data presented in this study are available in the article or Supplementary Material.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

Appendix A. Materials and Methods

Appendix A.1. Reagents/Chemicals Used in This Study

- Acetic acid glacial (Sigma, St. Louis, MO, USA);
- Ammonium Carbonate—$(NH_4)_2CO_3$ (Merck KGaA, Darmstadt, Germany);
- Bile bovine (Sigma, St. Louis, MO, USA);
- Bile salts (Sigma, St. Louis, MO, USA);
- Bile acid assay kit (Sigma, St. Louis, MO, USA);
- Bimuno daily (GOS) (Clasado Ltd., Reading, UK);
- Butyric acid (Sigma, St. Louis, MO, USA);
- Calcium chloride dihydrate—$CaCl_2(H_2O)_2$ (Carlo Erba Reagents, Barcelona, Spain)
- Calcium chloride hexahydrate—$CaCl_2(H_2O)_6$ (Sigma, St. Louis, MO, USA);
- Defibrinated sheep blood OxoidTM (Thermo Fischer Scientific, Waltham, MA, USA)
- Dipotassium hydrogen phosphate—K_2HPO_4 (Honeywell Fluka, Seelze, Germany);
- DL—lactic acid (Sigma, St. Louis, MO, USA);
- Fructooligosaccharides from chicory root (FOS) (Megazyme, Bray, Ireland);
- Glycerol—analytical grade (Fisher Scientific, Loughborough, UK);
- Hemin (Sigma, St. Louis, MO, USA);
- Hemoglobin (Sigma, St. Louis, MO, USA);
- Hydrochloric acid—HCl (Honeywell Fluka, Seelze, Germany);
- Inulin from chicory (Sigma-Aldrich, St. Louis, MO, USA);
- L-cysteine HCl (Sigma-Aldrich, St. Louis, MO, USA);
- Magnesium chloride hexahydrate—$MgCl_2(H_2O)_6$ (Panreac, Barcelona, Spain);
- Magnesium sulfate heptahydrate—$MgSO_4(H_2O)_7$ (Sigma, St. Louis, MO, USA);
- Molico skim milk powder—SKM (Nestlé S.A., Vevey, Switzerland);
- Na-p-tosyl-L-arginine methyl esther hydrochloride-TAME (Sigma, St. Louis, MO, USA);
- Ohly-GO® SoluMOS (Ohly, Hamburg, Germany);
- Pancreatin from porcine pancreas (Sigma, St. Louis, MO, USA);
- Pepsin from porcine gastric mucosa powder (Sigma, St. Louis, MO, USA);
- Peptone from animal tissue (Sigma, St. Louis, MO, USA);
- Phosphate buffered saline (Dulbecco A) OxoidTM (Thermo Fischer Scien-tific, Waltham, MA, USA);
- Potassium chloride—KCl (Honeywell Fluka, Seelze, Germany);
- Potassium dihydrogen phosphate—KH_2PO_4 (Merck KGaA, Darmstadt, Germany);
- Propionic acid (Sigma, St. Louis, MO, USA);
- Resazurin sodium salt (Sigma, St. Louis, MO, USA);
- Sodium chloride—NaCl (Honeywell Fluka, Seelze, Germany);

- Sodium hydrogen carbonate—$NaHCO_3$ (Panreac, Barcelona, Spain);
- Sodium hydroxide—NaOH (LabChem, Zelienople, PA, USA);
- Sulfuric acid—H_2SO_4 (Honeywell Fluka, Seelze, Germany);
- Trichloroacetic acid—TCA (Sigma, St. Louis, MO, USA);
- Tris(hydroxymethyl)aminomethane hydrochloride (Merck KGaA, Darmstadt, Germany)
- Tween 80 (Sigma, St. Louis, MO, USA);
- UL-250® (*Saccharomyces boulardii*)(Biocodex, Gentilly, France);
- Vitamin K1 (Sigma, St. Louis, MO, USA);
- Yeast extract (Sigma, St. Louis, MO, USA).

Appendix A.2. Culture Media

- Columbia agar base—CBA (Liofilchem, Roseto degli Abruzzi, Italy);
- de Man, Rogosa and Sharpe agar—MRSA (Biokar Diagnostics, Allonne, France);
- MacConkey agar—MCA (Biolife, Milan, Italy);
- Muller-Hinton broth (Biokar diagnostics, Allonne, France);
- Tryptic soy broth (Biokar diagnostics, Allonne, France).

Appendix A.3. Apparatus Used in This Study

- 1 kDa molecular weight cut-off regenerated cellulose dialysis tubing Spec-tra/Por® 6 (Spectrum, New Brunswick, NJ, USA);
- Agilent 1260 II series HPLC (Agilent, Santa Clara, CA, USA);
- Alpha 2–4 LSC plus model (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany);
- Anaerobic cabinet, Whitley A35 workstation (Don Whitley Scientific, Bingley, UK);
- FerMac 260 pH controller (Electrolab Biotech Ltd., Gloucestershire, UK);
- Heraeus™ Megafuge™ 16R Centrifuge (Thermo Fischer Scientific, Waltham, MA, USA)
- Ion-exclusion Aminex HPX-87H column (Biorad, Hercules, CA, USA);
- Mixwel® laboratory blender (Alliance Bio Expertise, Guipry, France);
- MR Hei-Tec magnetic stirrer (Heidolph Instruments GmbH & CO. KG, Schwabach, Germany)
- MST magnetic stirrer (Velp Scientifica, Usmate Velate, Italy)
- Oxoid™ AnaeroGen™ 2.5 L sachet (Thermo Fischer Scientific, Waltham, CA, USA);
- Oxoid™ AnaeroJar™ 2.5 L (Thermo Fischer Scientific, Waltham, CA, USA);
- Reax top vortex (Heidolph Instruments GmbH & CO. KG, Schwabach, Germany);
- Refrigerator Beko RSNE445E33WN (Beko, Istanbul, Turkey);
- Sension+ 9663 Ammonium Ion Selective Electrode (ISE) (Hach, CO, USA);
- Synergy H1 Hybrid Multi-Mode Reader (BioTek, Winooski, VT, USA);
- UV-1900 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan);
- Tamper proof specimen 1-L containers (Sigma, St. Louis, MO, USA).

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