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In vitro modulation of gut microbiota by whey protein to preserve intestinal health

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The effect of several types of whey milk – cow, sheep, goat and a mixture of them (60 : 20 : 20, respectively) – was assessed in the human gut microbiota. The prebiotic potential of these substrates was evaluated through *in vitro* gastrointestinal digestion following faecal batch culture fermentations (mimicking colonic fermentation) for 48 hours, using faeces from normal-weight (NW) and obese (OB) donors. Throughout the fermentation process, pH, gas production, short chain and branched fatty acids (SCFA-BCFA) were measured, as well as the changes of microbiota using qPCR. The pH decreased in all whey samples during the fermentation process. Gas production was higher in all whey samples than in controls, especially at 12 hours ($p < 0.05$). The diversity of SCFA and BCFA production was significantly different between the donors, in particular cow and mixed whey. Whey milk had a strong prebiotic effect on the gut microbiota of NW and OB donors, showing a significant increase of *Bifidobacterium* ($p < 0.05$) with cow, sheep and mixed whey and increase in the *Lactobacillus* group, particularly in OB donors. Bacteria associated with obesity did not show an increase in any of the groups of donors. Therefore, supplementing a diet with these types of whey can selectively stimulate the growth of probiotic bacteria, enhancing SCFA production, which could improve intestinal disorders. In addition, it may be an interesting approach to the prevention of overweight and obesity and related diseases. Whey milk has a potent prebiotic effect. It can selectively stimulate desirable bacteria and SCFA profile, in both OB and NW donors, contributing to improved intestinal health and reducing obesity.

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Introduction

From 1980 to 2014 the global obesity rate has doubled. In 2014, more than 1.9 billion adults were overweight; of these over 600 million were obese (OB).[1] If the observed trends persist, by 2030 the number could rise to a total of 1.12 billion, reaching 20% of the world's adult population.[2] Its prevalence is increasing and its importance to public health is considerable because it is associated with a higher risk of type 2 diabetes, coronary heart disease, certain cancers[3] and a shorter life expectancy. Obesity is thought to be a multifactorial disorder. Genetic factors may predispose individuals to overweight and obesity, but a reduction in physical activity, an increase in energy intake (basically fat-rich and energy-dense foods) and an inadequate life style could lead to an imbalance between energy intake and expenditure, resulting in weight gain.[4]

The gut microbiota has an important role in the regulation of energy homeostasis and energy intake. It has also been considered the "forgotten organ" because it affects the absorption and fermentation of nutrients, having a direct association with health and metabolism.[5] It has been reported a specific profile of bacteria in OB and normal-weight (NW) subjects. OB microbiota is associated with a low diversity of bacteria population with an increase of *Firmicutes* and a decrease in *Bacteroidetes* in OB animals and humans.[6–9] Nevertheless, NW is associated with an increase in *Bifidobacterium animalis*, *Methanobrevibacter smithii* and phylum *Bacteroidetes*.[10] An important mechanism that could explain the relationship between the gut microbiota and body mass is that bacteria could improve the host's ability to extract energy from the diet and store it in the adipose tissue.[7–9] Treatments to manipulate the gut microbiota could be an interesting approach to prevent and treat overweight and obesity. Furthermore, microbiota has a direct relationship with gut health. Low-grade inflammation in OB people has been reported, which is mainly caused by altered microbiota.[11]

Taking the importance of microbiota into account, recent research has focused on the development and study of ingredients able to improve microbiota composition and hence intestinal health.[12–14] In addition, ingredients that could also

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promote a satiating effect, may contribute to the reduction of obesity.[15,16] Thus, ingredients with these two characteristics (improving microbiota and enhancing satiety) have raised interest among the research community. One could be whey milk, as it is a by-product that is easy to obtain from cheese production with high-quality proteins and nutritional value. Whey is the liquid fraction obtained during the coagulation of milk in cheese production. This by-product is mainly composed of proteins (1%) and lactose (5%), having both different and interesting effects on satiety and gut protection through increasing satiety and being fermented by colonic bacteria, respectively. The whey protein (WP) fraction consists of mainly β-lactoglobulin, α-lactalbumin, immunoglobulins, serum albumin, lactoferrin, lactoperoxidase and a minor component corresponding to glycomacropeptide. All these proteins are demonstrated to affect satiety by reducing food intake,[17,18] stimulating satiating gut hormone production, including cholecystokinin (CCK)[19] and GLP-1[20] and slowing stomach emptying.[21] Besides, many authors suggest a positive effect of whey milk (specifically protein and bioactive peptides) on gut health, providing non-immune defence against pathogenic bacteria,[22–25] as well as a probiotic effect.[26–28] Also, the main fermentable substrates are lactose, peptides and amino acids from whey which produce short chain fatty acids (SCFAs) and branched chain fatty acids (BCFAs).[29] SCFAs are important mediators of the interaction between gut microbes and hosts. Butyrate acts as an energy source of colonocytes; propionate is transported to liver, where it has a role in gluconeogenesis; and acetate is used in lipogenesis. Whey is also rich in lactose, which is used as a substrate by beneficial bacteria, such as lactobacilli and bifidobacteria.[30] They produce lactate that could improve intestinal health, preventing the growth of other pathogens, such as *Salmonella* or *E. coli.*[31] WP can be affected by the type of whey (acid or sweet), milk source (cow, sheep, goat *etc.*), animal feeding and breed, lactation period and quality of cheese processing.[32] Taking everything into account, in this study, we assessed the qualitative and quantitative differences of the gut microbiota in response to different types of whey milk. The compositions of the different bacterial genera including SCFA production were investigated. In the present study stirred and non-pH controlled faecal batch culture fermentation were used. This could have implications for the design of new prebiotic and satiating ingredients based on whey milk and future applications in reducing overweight and obesity prevalence and incidence.

Materials and methods

Whey milk samples

Four types of mammalian species of whey milk were used in this study: Friesian cow, Segureña sheep, Murciano-Granadina goat and a mixture of the above (60% cow, 20% sheep and 20% goat). All these types of whey milk were provided by Palancares Alimentación S.L., a local cheese factory (Murcia, Spain). Each kind of whey was lyophilized and stored away from light and humidity. Fig. 1 illustrates the experimental design of the present study, showing the different stages performed.

In vitro digestion of whey (from mouth to the small intestine)

Simulated gastrointestinal digestion was performed using the protocol described previously by Minekus *et al.*[33] which consisted of 3 phases: oral, gastric and intestinal. It started with 3 g of lyophilized whey mixed with 3 mL of simulated salivary fluid (SSF) and α-amylase (150 U mL^{-1}) (50 : 50 w/v). The mixture was maintained for 2 minutes in a water bath at 37 °C and 60 strokes per minute. During the gastric phase, simulated gastric fluid (SGF) was added to the oral phase volume (50 : 50 w/v), the pH was adjusted to 3 with the addition of 1 M HCL and then pepsin was added (1000 U mL^{-1}). Samples were incubated under the same conditions as for the oral phase for 2 hours. Finally, in the intestinal phase, simulated intestinal fluid (SIF) was added to gastric digestion (50 : 50 w/v), the pH was adjusted to 7 using 6 M NaOH, and then bile salts (20 mM) and pancreatin (200 U mL^{-1}) were added. This mixture was incubated under the same conditions as before for 2 hours. At the end, samples were stored on ice, to stop enzymatic activity, before batch culture fermentation. All reagents for *in vitro* digestion were purchased from Sigma-Aldrich.

Faecal sample preparation and stabilization

Human microbiota samples were obtained from the faeces of 3 NW and 3 OB volunteers. Donors were between 25 and 45 years, non-smokers, had not taken antibiotics, probiotics or prebiotics in the last 3 months, and were not pregnant. In addition, the weights of the volunteers were stable (<2 kg change in the past 3 months) and without any clinical history of intestinal disease or metabolic diseases. All experiments were performed in compliance with the relevant laws and institutional guidelines. The Ethical Research Committee of The University of Murcia approved the study protocol, and written informed consent was obtained from each volunteer before donating the faeces.

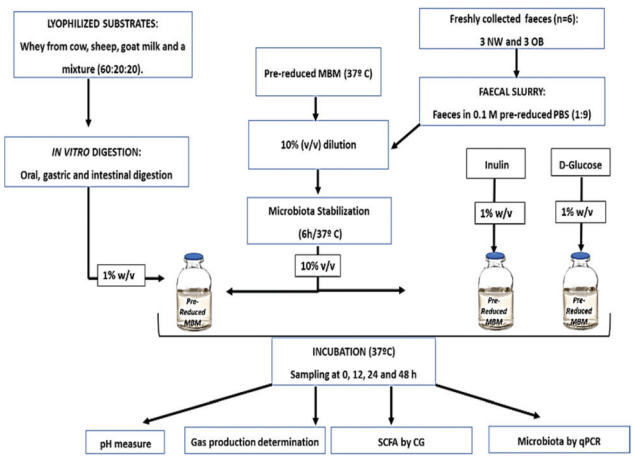

Fig. 1 Flow diagram of the experimental design showing the different stages carried out in the present study.

Faecal samples were kept at 4 °C under anaerobic conditions and processed within 1 hour after collection to maintain their microbial community composition in optimal conditions.[34] The stabilization of faeces was carried out with a dilution of 1 : 9 (w/v) with pre-reduced phosphate buffered saline (PBS), homogenized in a stomacher and inoculated in wheaton bottles with pre-reduced minimal basal medium (MBM) (10% v/v). It was incubated under anaerobic conditions for 6 hours. MBM was previously prepared containing per litre, peptone water (2 g), yeast extract (2 g), NaCl (0.1 g), KH_2PO_4 (0.04 g), K_2HPO_4 (0.04 g), $MgSO_4{\cdot}7H_2O$ (0.01 g), $CaCl_2{\cdot}6H_2O$ (0.01 g), $NaHCO_3$ (2 g), L-cysteine (0.5 g), bile bovine (0.5 g), Tween 80 (2 ml) and 4 ml of resazurin solution (0.025%; w/v). Resazurin was added as an anaerobiosis/redox potential indicator.[35] Any manipulation of samples containing microbiota throughout the study was conducted in a biological safety cabinet of vertical flow. Digested whey, inulin as the prebiotic control (Alfa Aesar, Ward Hill, MA)[13] and D-glucose as the non-prebiotic control (Sigma-Aldrich)[13,36] were assessed at 1%. The stabilized microbiota was inoculated at 10% v/v in every sample and maintained under anaerobic conditions for 48 h. At 0, 12, 24 and 48 hours,[13,37,38] pH (Crison, Germany), gas pressure (Wika Instruments, S.A.U., Barcelona, Spain),[39] SCFA and bacteria quantification were measured. All fermentation samples were assessed in duplicate.

Quantification of SCFAs by gas chromatography

The SCFA extraction of digested samples and controls was performed prior to analyses: 100 μL of cell free supernatant was added to 650 μL of a mixture of extra pure formic acid (Scharlau, Spain) (20%), methanol of HPLC gradient grade (J.T. Baker, The Netherlands) and 2-ethyl butyric acid (Merck, Germany) (internal standard, 2 mg ml^{-1} in methanol) at a ratio of 1 : 4.5 : 1. The mixtures were homogenized by vortexing and filtered through a 13 mm (diameter), 0.22 μm (pore size) PTFE filter (VWR International, USA). Then, 1 μL of samples were analysed using a gas chromatograph (Agilent 7890A) equipped with a flame ionization detector and a Nukol™ GC-column (30 m × 0.25 × 0.25 μm) following the method proposed by Zhao *et al.*[40] The chromatographic conditions are shown in Table 1. The calibration curve was obtained using a volatile acid standard mix (Supelco, Bellefonte, PA, USA) of acetic, propionic, butyric, i-isobutyric, valeric, i-valeric, caproic, i-caroic and heptanoic acid, containing a concentration of 10, 5, 2, 1, 0.5 and 0.25 mM. The area of each peak of SCFAs was integrated using Agilent Chemstation Operation software (Santa Clara, USA), and concentrations were calculated by comparing their peak areas with those of the standard and were expressed in mM. Every sample was run in quadruplicate.

Table 1 Chromatographic conditions

Equipment parameters	Conditions
Elution method	Constant pressure injection
Gas flux	He_2, 25 ml min^{-1} (carrier) Air, 400 ml min^{-1} H_2, 30 ml min^{-1}
Injection method	Splitless
Volume of injection	2 μl
Oven temperature	80 °C
Temperature ramp	80 °C for 5 min 5 °C min^{-1} until 185 °C
Injector temperature	220 °C
Detector temperature	220 °C
Injector pressure	58.99 kPa

DNA extraction and analysis of faecal microbiota from fermented samples

DNA extraction from faecal bacteria was based on the protocol described by Boon *et al.*[41] All materials and water used in this phase were autoclaved twice and were RNase-free. The DNA concentration of each sample was measured at a 260/280 nm ratio, using a NanoDrop-100 spectrophotometer (Thermo Fisher Scientific, Villebon sur Yvette, France). Quantitative real-time PCR (qPCR) was used to characterize the microbiota changes, using specific primers for each bacterial group. The qPCR protocol was carried out in a 96-well CFX96 Real-Time PCR thermocycler and detection system (Bio-Rad, Madrid, Spain). Reactions were performed in a volume of 25 μL per reaction, containing 1 μL of template DNA, 12.5 μL of SensiMix™ SYBR No-ROX (Bioline, London, UK), 0.5 μL of both specific primers (0.2 mM) and 10.5 μL of nuclease-free water (AppliChem, Darmstadt, Germany). Every sample was run in quadruplicate. The amplification program was run at 95 °C for 10 minutes for DNA initial denaturation and enzyme activation, 40 cycles of denaturation (95 °C for 15 seconds), annealing ((Table 2) 60 °C for 30 seconds) and extension (72 °C for 45 seconds). The melting curve was obtained from 65 °C to 95 °C, with an increase of 0.5 °C every 0.5 seconds. The fluorescent products were detected in the last step of each cycle. The bacteria concentration from each sample was calculated by comparing the cycle threshold (Ct) values obtained from the standard curves. These standard curves were created using serial 10-fold dilution of pure culture DNA corresponding to 10^2 to 10^8 cell equivalents per ml (genome equivalents per mL). The conversion of the number of bacterial DNA in samples determined by qPCR to theoretical genome equivalents, required the assumption that similarities exist between the amplicon size and the 16S ribosomal RNA gene copy number for each microbial group.[42] The following bacterial groups were analysed in this study: *Bacteroides* (using *Bacteroides thetaiotaomicron*, DSMZ 2079 as standard), *Bifidobacterium* (*Bifidobacterium longum*, CECT 4503), *Firmicutes* (*Clostridium leptum*, DSMZ 753), *Enterobacteriaceae* (*Escherichia coli*, NUTBRO Collection), *Lactobacillus* (*Lactobacillus gasseri*, DSMZ 20077) and total bacteria (*Bifidobacterium longum*, CECT 4503).

Statistical analysis

Statistical data processing was performed using the statistical program Statistical Package for the Social Sciences (SPSS) v.19.0. Prior to analysis, normality and homoscedasticity were

Table 2 Specific primers used in the study to target different bacterial groups

Bacterial group	Target	Primers	Ann. temp. (°C)	Reference
Total bacteria	16S	Fwd: GTGSTGCAYGGYYGTCGTCA Rv: ACGTCRTCCMCNCCTTCCTC	60	Fuller *et al.*[43]
Bacteroides–Prevotella	16S	Fwd: GAGAGGAAGGTCCCCCAC Rv: CGKACTTGGCTGGTTCAG	60	Layton *et al.*[44]
Bifidobacterium	16S	Fwd: GATTCTGGCTCAGGATGAACGC Rv: CTGATAGGACGCGACCCCAT	60	Gueimonde *et al.*[45]
Enterobacteriaceae	16S	Fwd: TGCCGTAACTTCGGGAGAAGGCA Rv: TCAAGGACCAGTGTTCAGTGTC	60	Matsuda *et al.*[46]
Lactobacillus	16S	Fwd: AGCAGTAGGGAATCTTCCA Rv: CATGGAGTTCCACTGTCCTC	60	Rinttila *et al.*[47]
Firmicutes	16S	Fwd: GGAGYATGTGGTTTAATTCGAAGCA Rv: AGCTGACGACAACCATGCAC	60	Guo *et al.*[48]

analyzed using the Shapiro–Wilk and Levene tests, respectively, setting the level of significance to $p < 0.05$. Results are expressed as means of four measures and SD in the case of bacterial metabolites and SEM in the case of microbiota population. To determine significant differences between the mean values of donors (pH, gas and SCFA production, and bacterial populations) *t*-tests were used ($p < 0.05$). To establish differences between the substrates and times of fermentation (pH, gas and SCFA production, and bacterial populations) one-way analysis of variance (ANOVA) and a subsequent *post-hoc* Tukey test were carried out ($p < 0.05$).

Results

The aim of this study was to evaluate pH, gas production, SCFA and microbiota composition in whey samples after *in vitro* digestion followed by batch culture fermentation of the faeces of NW ($n = 3$) and OB ($n = 3$) donors. Results were evaluated by comparing both groups.

pH values and gas production during fermentation

pH values are shown in Table 3. pH values decreased significantly ($p < 0.05$) along fermentation with respect to time 0 in all fermentation times and samples of whey. There were significant differences between different fermentation times, although there were slight differences between substrates or groups of donors. The highest reductions were found from 0 to 12 h in all samples. We only found significant differences ($p < 0.05$) at the initial time of fermentation between donors, corresponding to an initial value of 6.77 units of pH for OB and 6.44 for NW donors.

Gas evolution data are presented in Table 4. The highest gas production was recorded in all whey samples with respect to controls, especially at 12 hours ($p < 0.05$), being less pronounced at subsequent times of fermentation. This increase was marked in the case of cow whey milk, showing the highest increment (72.41 kPa) at 12 h of fermentation which was compared with the rest of ingredients in NW donors. There were also significant differences ($p < 0.05$) in the increase of gas production at 12 and 24 h when the 2 groups of donors were compared, corresponding to the largest gas increment to the NW group.

Table 3 Decrease in pH (units) of batch culture fermentation, after 48 h, of four types of whey milk (cow, sheep, goat and mixture) (NW ($n = 3$) and OB ($n = 3$))

Time	Cow	Sheep	Goat	Mixture
Normal-weight				
0 h	$6.44^{d} \pm 0.02$	$6.44^{d} \pm 0.02$	$6.44^{d} \pm 0.02$	$6.44^{c} \pm 0.02$
12 h	$4.90^{c} \pm 0.03$	$4.93^{c} \pm 0.04$	$5.06^{c} \pm 0.05$	$5.16^{b} \pm 0.02$
24 h	$4.29^{b} \pm 0.03$	$4.41^{b} \pm 0.08$	$4.54^{b} \pm 0.02$	$4.35^{a} \pm 0.04$
48 h	$3.88^{a} \pm 0.07$	$3.95^{a} \pm 0.05$	$3.99^{a} \pm 0.03$	$4.07^{a*} \pm 0.03$
Obese				
0 h	$6.77^{d*} \pm 0.04$	$6.77^{d*} \pm 0.04$	$6.77^{d*} \pm 0.04$	$6.77^{d*} \pm 0.04$
12 h	$4.88^{c} \pm 0.02$	$4.94^{c} \pm 0.04$	$5.07^{c} \pm 0.01$	$5.02^{c} \pm 0.03$
24 h	$4.40^{b} \pm 0.04$	$4.99^{b} \pm 0.02$	$4.65^{b} \pm 0.01$	$4.35^{b} \pm 0.04$
48 h	$3.92^{a} \pm 0.02$	$3.98^{a} \pm 0.02$	$4.04^{a} \pm 0.01$	$3.97^{a} \pm 0.01$

Results are expressed as mean ± S.D. Letters a, b, c, and d denote significant differences for a given variable between the different times of fermentation per whey milk ($p < 0.05$). * denotes significant differences for a given variable between the different groups of donors ($p < 0.05$).

SCFA and BCFA production during fermentation

The total concentration (mM) of fatty acids (acetate, propionate, i-butyric, butyrate, i-valeric, valeric, i-caproic, caproic and heptanoic acid) during the fermentation of several types of whey milk is presented in Fig. 2A. The concentration of total fatty acids increased significantly upon fermentation, reaching values near 50 mM in both groups of donors after 48 h of cow, sheep and mixed whey fermentations. Some statistically significant differences ($p < 0.05$) were found when fatty acid concentrations from both types of donors were compared. Specifically, NW showed a higher concentration than OB after 48 h of cow and sheep whey fermentations; meanwhile, OB produced more SCFA after 24 h of mixed whey fermentation (43.69 and 25.39 mM, respectively). The molar proportions of the main SCFA (acetate, propionate, butyrate) and minor SCFA (i-butyric, i-valeric, valeric, i-caproic, caproic and heptanoic acid) after fermentation are presented in Fig. 2B.

Table 4 Increment of gas (kPa) produced by faecal bacteria from human donors (NW (n = 3) and OB (n = 3)) during fermentation (12, 24 and 48 h) with four types of whey milk (cow, sheep, goat and mixture)

Time	Cow	Sheep	Goat	Mixture
ΔGas (kPa); normal-weight				
12 h	$72.41^{a*} \pm 1.12$	$65.98^{b*} \pm 2.34$	$63.36^{b*} \pm 3.83$	$66.38^{b*} \pm 3.08$
24 h	$20.66^{a*} \pm 1.18$	$23.91^{a*} \pm 3.73$	$19.80^{a*} \pm 1.72$	$19.93^{a*} \pm 2.87$
48 h	$5.06^{ab*} \pm 0.25$	$5.63^{a*} \pm 0.18$	$4.66^{b} \pm 0.47$	$4.66^{b} \pm 0.27$
ΔGas (kPa); obese				
12 h	$45.05^{a} \pm 6.75$	$45.45^{a} \pm 5.05$	$49.21^{a} \pm 2.60$	$54.20^{a} \pm 3.69$
24 h	$7.40^{b} \pm 1.10$	$7.43^{b} \pm 1.12$	$9.90^{a} \pm 0.81$	$7.35^{b} \pm 1.24$
48 h	$3.90^{a} \pm 0.42$	$4.28^{a} \pm 0.82$	$4.31^{a} \pm 0.69$	$4.31^{a} \pm 0.76$

Results are expressed as mean ± S.D. Letters a, b, c, and d denote significant differences for the same time of fermentation and whey milk ($p < 0.05$). * denotes significant differences for a given variable between the different groups of donors ($p < 0.05$).

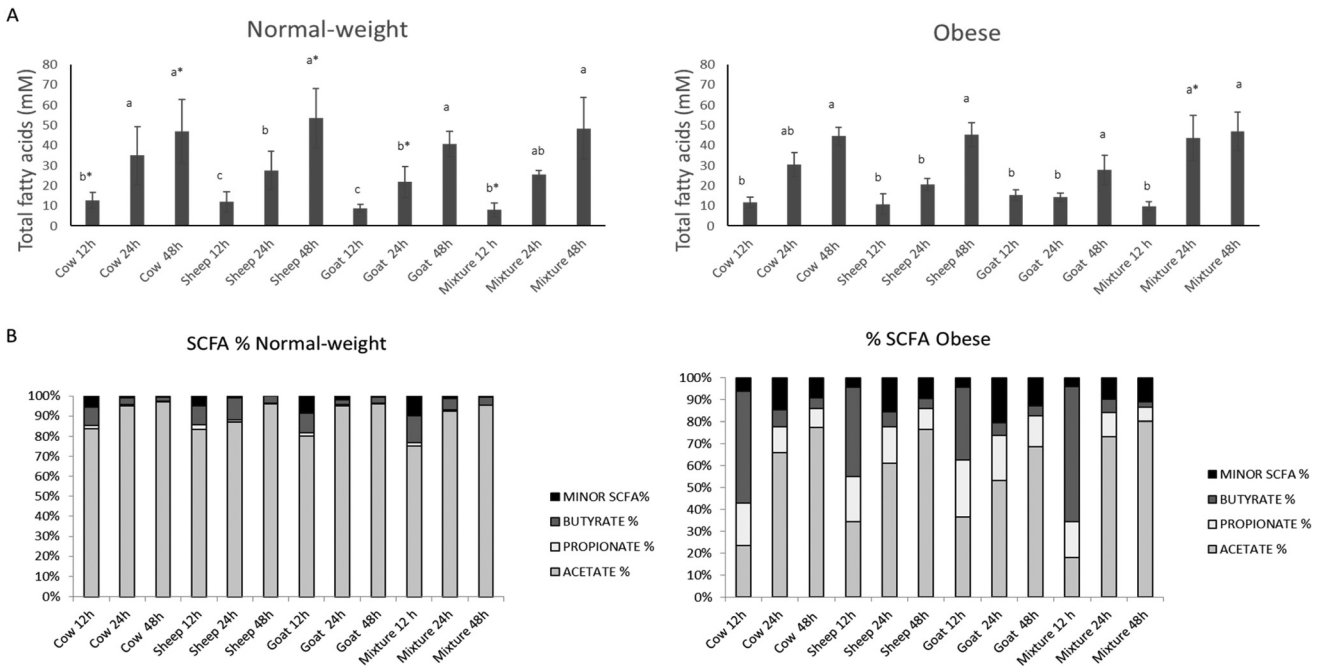

Fig. 2 Short chain fatty acid production along fermentation. (A) Total concentration (mM) of fatty acids (acetate, propionate, i-butyric, butyrate, i-valeric, valeric, i-caproic, caproic and heptanoic acid) for 48 h of fermentation with substrates (cow, sheep, goat and mixture whey milk) in NW (n = 3) and OB (n = 3) groups. Letters a, b, c, and d denote significant differences for a given variable between the different times of fermentation per whey milk ($p < 0.05$). * denotes significant differences for a given variable between the different groups of donors ($p < 0.05$). (B) Molar proportions (%) of the three major fatty acids (acetate, propionate and butyrate) and minor fatty acids (i-valerate, i-butyrate, i-caproate, valerate, caproate and heptanoic acid) for 48 h of fermentation with substrates (cow, sheep, goat and mixture whey milk) in NW (n = 3) and OB (n = 3) groups.

Acetate and propionate showed a tendency to increase with fermentation time, mainly acetate in the case of the OB group. In contrast, butyrate showed a reduction in the relative proportion throughout the fermentation process. Acetate was the most relevant contributor to SCFAs in the NW group, especially at the end of fermentation. Nevertheless, propionate, butyrate and minor fatty acids were the most important ones in the OB group, showing statistically significant differences ($p < 0.05$) between both groups for any whey and time. Comparing the NW and OB SCFA productions, we found statistically significant differences ($p < 0.05$) in the case of all SCFAs. The acetate level was significantly higher in NW for the cow and mixed whey at 12 h, while for sheep a high increase (other than for OB) was at 48 h, presenting a relative proportion of 95.99% *versus* 76.66%, respectively. In relation to butyrate, the NW and OB donors showed a marked decrease ($p < 0.05$) over the course of fermentations in all samples of whey. We found statistically significant differences ($p < 0.05$) after comparing both types of donors, obtaining higher amounts of butyrate in the OB volunteers. It is worth noting the differences after 12 hours of mixed whey fermentation, obtaining 61.2% and 13.4% of butyrate in OB and NW, respectively. In all samples of whey, butyrate showed a substantial reduction near 90%, from 12 to 48 hours. Propionate levels

were also significantly higher in the OB than NW donors in all samples of whey. In every whey and donor, propionate increased slightly over fermentations, but its relative proportion decreased due to the high increase in acetate. In relation to the molar proportions of minor fatty acids, i-valerate was the main contributor. We found statistically significant higher proportions ($p < 0.05$) in OB donors than in NW donors, mainly at 24 h of fermentations. The greatest differences at this time were found for goat whey (20.35% in OB *versus* 1.92% in NW donors). At 48 h there were also significant increases in OB donors compared to NW donors, especially for mixed whey (10.85% *vs.* 0.64%).

Analysis of faecal microbiota of NW and OB donors after fermentation

The original microbial population (time 0 h) of both groups of donors is shown in Fig. 3. As mentioned before, OB and NW microbiota differ. This study reinforced this theory, reporting differences in the initial microbiota composition of both groups of donors. Significant differences ($p < 0.05$) were found in *Firmicutes*, *Bacteroides* and *Lactobacillus* being higher in OB than in NW. In the case of lactobacilli, the initial gut difference was marked: 3.64 and 5.01 log genome equivalents per mL in NW and OB, respectively.

Initial microbiota and changes in the bacteria population after 12, 24 and 48 h of fermentation with the different types of whey milk are shown in Table 5. The total bacteria population showed statistically significant differences ($p < 0.05$) at 48 h of cow and sheep whey fermentation when both donors were compared. Focusing on the total bacteria evolution over time in each type of donor, we found a significant decrease ($p < 0.05$) from 12 to 48 h in the sheep whey fermentation in NW; significant differences with respect to controls at time 12 h for cow, sheep and mixed whey occurred (data not shown). In the case of OB, we found a significant increase in the total bacteria after 12 h of cow whey fermentation. However, at 12 h, all whey types showed higher values than the controls (data not shown).

The *Lactobacillus* group showed a decreasing tendency in all samples from NW donors, although without any significant differences. Nevertheless, in the OB group an opposite effect was found, with an increase near 2 log genomic equivalents per mL at time 48 h in the case of cow and sheep (7.1 ± 0.34 and 7.06 ± 0.34, respectively). All samples of whey during fermentation were significantly different in the OB than in the NW group (except in the case of sheep whey at 12 h), as there was a higher number of lactobacilli in the OB.

The *Bifidobacterium* group showed an approximate increase of 1 log genomic equivalent per mL in all whey and donors, obtaining statistically significant differences in some cases. In NW donors, we found a significant increase in sheep and mixed whey from 0 h to 24 or 48 h, respectively. Furthermore, significantly higher values of bifidobacteria were observed in every type of whey and at every time compared to the controls (data not shown). In OB donors, we found significant increases with respect to the initial time of fermentation in cases of cow and mixed whey (12, 24 and 48 h) with differences compared to controls at 24 and 48 h. Differences between both types of donors were not found.

The *Bacteroides* group in NW showed a significant decrease throughout the fermentation (cow, sheep and goat). In OB, the bacteria population decreased over time, although not significantly. However, we found significantly higher values of *Bacteroides* in OB than NW in most times and whey samples. The *Firmicutes* group data did not show significant increases throughout fermentation, remaining stable in both OB and NW donors, although the initial value was significantly higher in OB. *Enterobacteriaceae* in NW show a slight increment in cow and sheep whey at 12 h and a reduction at 24 h. In OB increases occurred at 48 h but were not statistically significant between times of fermentation. However, significant differences occurred between the groups of donors, indicating increases in cow and goat whey at 24 and 48 h, and mixed and sheep whey at the end of the fermentation.

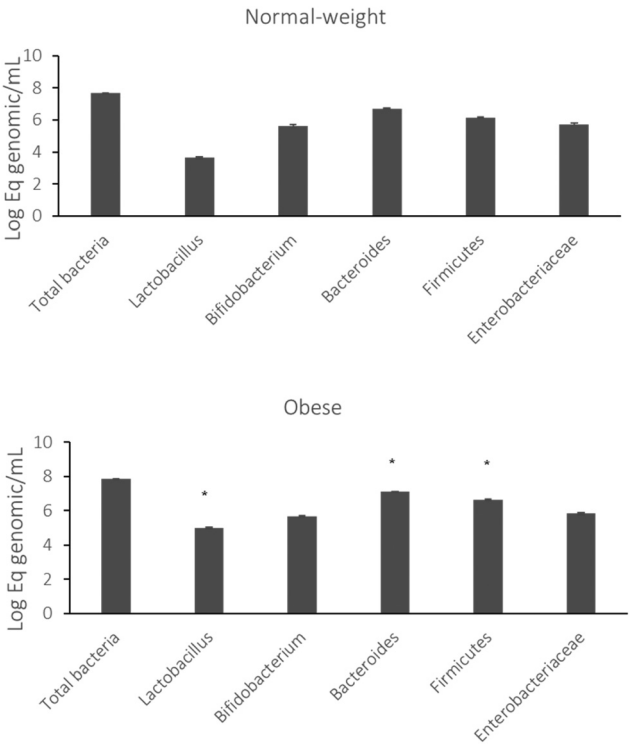

Fig. 3 Initial microbial population (logarithm genome equivalents per mL) of normal-weight ($n = 3$) and obese ($n = 3$) groups of donors. Bars represent means ± S.E.M. of quadruplicate cultures. * denotes significant differences for different groups of donors ($p < 0.05$).

Discussion

Whey milk is mainly composed of protein and lactose, and both can act as useful substrates for bacteria and approximately 10% of protein intake reaches the human colon, although it depends on the amount and kind of protein

Table 5 Bacterial populations (log genomic equivalents per mL) from NW (n = 3) and OB (n = 3) donors after 48 h of fermentation using several types of whey (cow, sheep, goat and mixture)

	Time	Total bacteria	Lactobacillus	Bifidobacterium	Bacteroides	Firmicutes	Enterobacteriaceae
Normal-weight							
Cow	0	7.68 ± 0.05	3.64 ± 0.33	5.62 ± 0.13	6.71^{a} ± 0.15	6.16 ± 0.19	5.73^{b} ± 0.13
	12	8.01 ± 0.16	3.66 ± 0.59	6.12 ± 0.55	6.45^{ab} ± 0.17	6.30 ± 0.26	6.40^{a} ± 0.07
	24	7.63 ± 0.19	3.69 ± 0.33	6.79 ± 0.13	6.07^{bc} ± 0.14	6.25 ± 0.26	5.78^{b} ± 0.20
	48	7.49 ± 0.04	3.53 ± 0.37	6.33 ± 0.17	5.65^{c} ± 0.11	5.95 ± 0.19	5.44^{b} ± 0.17
Sheep	0	7.68^{ab} ± 0.05	3.47 ± 0.26	5.60^{b} ± 0.05	6.81^{a} ± 0.13	6.10 ± 0.06	5.71^{b} ± 0.10
	12	7.98^{a} ± 0.16	4.28 ± 0.41	5.95^{ab} ± 0.41	6.14^{b} ± 0.11	6.26 ± 0.19	6.37^{a} ± 0.10
	24	7.66^{ab} ± 0.18	3.89 ± 0.30	6.79^{a} ± 0.17	5.95^{bc} ± 0.22	6.33 ± 0.35	5.77^{ab} ± 0.29
	48	7.39^{b} ± 0.07	3.16 ± 0.48	6.54^{a} ± 0.16	5.53^{c} ± 0.11	5.87 ± 0.21	5.58^{b} ± 0.08
Goat	0	7.68 ± 0.05	3.64 ± 0.33	5.62 ± 0.13	6.71^{a} ± 0.15	6.16 ± 0.78	5.73 ± 0.13
	12	7.76 ± 0.23	3.31 ± 0.43	6.05 ± 0.51	6.27^{ab} ± 0.23	5.84 ± 0.35	6.00 ± 0.21
	24	7.65 ± 0.13	3.14 ± 0.30	6.51 ± 0.13	6.01^{ab} ± 0.10	5.78 ± 0.24	5.92 ± 0.21
	48	7.77 ± 0.12	3.18 ± 0.37	6.76 ± 0.19	5.71^{b} ± 0.11	5.87 ± 0.09	5.76 ± 0.09
Mixture	0	7.68 ± 0.05	3.64 ± 0.21	5.62^{b} ± 0.13	6.71 ± 0.15	6.16 ± 0.78	5.73 ± 0.13
	12	8.02 ± 0.17	3.59 ± 0.30	6.26^{a} ± 0.18	6.21 ± 0.14	6.45 ± 0.27	6.36 ± 0.23
	24	7.80 ± 0.19	3.73 ± 0.22	6.65^{a} ± 0.16	5.94 ± 0.29	6.03 ± 0.26	5.85 ± 0.40
	48	7.70 ± 0.18	3.15 ± 0.20	6.44^{a} ± 0.11	5.90 ± 0.22	6.20 ± 0.36	5.61 ± 0.20
Obese							
Cow	0	7.85^{b} ± 0.09	5.01^{b}* ± 0.60	5.64^{b} ± 0.19	7.09* ± 0.04	6.63* ± 0.18	5.81 ± 0.19
	12	8.44^{a} ± 0.12	5.77^{ab}* ± 0.66	6.47^{a} ± 0.34	7.11 ± 0.27	6.83 ± 0.32	6.73 ± 0.35
	24	8.07^{ab} ± 0.20	6.05^{ab}* ± 0.37	6.70^{a} ± 0.16	6.89* ± 0.24	6.31 ± 0.34	6.73* ± 0.33
	48	8.1^{ab}* ± 0.10	7.10^{a}* ± 0.34	6.65^{a} ± 0.11	6.68 ± 0.32	6.23 ± 0.34	6.39* ± 0.30
Sheep	0	7.85 ± 0.97	5.01^{b}* ± 0.60	5.64^{b} ± 0.19	7.09* ± 0.44	6.63* ± 0.18	5.81 ± 0.16
	12	8.29 ± 0.28	5.46^{ab} ± 0.24	6.31^{ab} ± 0.24	7.02 ± 0.44	6.74 ± 0.31	6.70 ± 0.46
	24	8.07 ± 0.20	6.07^{ab}* ± 0.25	6.57^{a} ± 0.19	6.84* ± 0.29	6.13 ± 0.33	6.75 ± 0.36
	48	8.36* ± 0.14	7.06^{a}* ± 0.34	6.62^{a} ± 0.12	6.68* ± 0.31	6.25 ± 0.36	6.52* ± 0.26
Goat	0	7.85 ± 0.09	5.01* ± 0.60	5.64^{b} ± 0.19	7.09* ± 0.04	6.63* ± 0.18	5.81 ± 0.16
	12	8.30 ± 0.14	5.46* ± 0.60	6.22^{ab} ± 0.25	7.02 ± 0.30	6.87 ± 0.31	6.72 ± 0.37
	24	8.10 ± 0.15	5.59* ± 0.37	6.43^{a} ± 0.23	6.86* ± 0.29	6.26 ± 0.27	6.8* ± 0.28
	48	7.81 ± 0.20	6.63* ± 0.51	6.29^{ab} ± 0.14	6.71* ± 0.26	5.88 ± 0.44	6.51* ± 0.26
Mixture	0	7.85 ± 0.09	5.01* ± 0.60	5.64^{b} ± 0.19	7.09* ± 0.04	6.63* ± 0.18	5.81 ± 0.16
	12	8.18 ± 0.15	5.43* ± 0.55	6.56^{a} ± 0.23	6.72 ± 0.25	6.59 ± 0.36	6.51 ± 0.30
	24	8.08 ± 0.12	5.92* ± 0.39	6.87^{a} ± 0.04	6.88* ± 0.25	6.30 ± 0.29	6.75 ± 0.30
	48	8.06 ± 0.08	6.68* ± 0.55	6.69^{a} ± 0.13	6.76* ± 0.26	6.27 ± 0.33	6.54* ± 0.25

Results are expressed as mean ± S.E.M. of quadruplicate cultures. Letters a, b, c, and d denote significant differences for a given substrate between the different times of fermentation ($p < 0.05$). * denotes significant differences for different donors ($p < 0.05$).

ingested.[49] In the present study, we have evaluated the prebiotic effect of several types of whey milk on human microbiota from NW and OB donors. Large amounts of milk proteins and peptides enter the large intestine where they are degraded into several peptides with well-known physiological effects, such as immunomodulatory and gastrointestinal activity (mineral-binding, anti-appetizing and antimicrobial activity).[50] Based on this, the use of milk whey as ingredient could produce bioactive peptides that can be released by the hydrolysis of digestive enzymes, the action of microorganism proteolytic enzymes[50] or through the hydrolysis of acido-lactic proteolytic bacteria such as *Lactococcus* sp. and *Lactobacillus* sp. in gut and other proteolytic bacteria able to degrade digested protein into peptides, such as *Bacteroides fragilis*, *Streptococcus*, *Propionibacterium*, *Clostridium*, *Bacillus* and *Staphylococcus*.[51,52] However, lactose contained in whey has a prebiotic effect for beneficial bacteria and it can be hydrolysed into galacto-oligosaccharides (GOS) by the enzyme β-galactosidase release by the gut microbiota (*Lactobacillus* sp., *Bifidobacterium* sp. and *E. coli*).[53]

A reduction in the pH may reflect SCFA and lactic acid production through the fermentation process, inhibiting the growth of pathogenic bacteria and promoting a probiotic effect on the gut induced by whey milk.[30,54,55] In this regard, *Bifidobacterium* and *Lactobacillus*, not only decrease the intestinal pH, but also enhance the lysozyme activity and facilitate the disruption of some pathogenic bacteria.[56] Gas production is an indicator of the activities of the total gut microflora. Rycroft *et al.*[57] demonstrated that the fermentation of GOS produced lactate and reduced gas production, probably because GOS stimulated the bifidobacteria population, demonstrating that there is an inverse relationship between the presence of *Bifidobacterium* and gas production. According to this, high gas production and low bifidobacteria levels at the beginning of the fermentation (from time 0 to 12 h) were found, whereas inverse results were found after 48 hours, in our study. Whey is composed of both, lactose and protein, and more proteolytic bacteria would contribute to the total gas production (*Clostridium* and *Streptococcus*). Moreover, *Bifidobacterium* can ferment lactate reducing its availability for gas-producing bac-

teria, such as *Clostridium* and sulphate-reducing bacteria.[58] The measurement of gas production could not be a precise predictor of what occurs in the gut environment since it cannot mimic the interactions of microorganisms and their metabolites and high variability can be observed.

Whey milk is a complex product that favours cross-feeding because metabolites produced by one type of bacterium serve as substrates for another. For example, lactate and acetate produced by *Bifidobacterium* can be converted to butyrate by another lactate-utilizing and butyrate-producing bacterium, such as *Firmicutes*,[59] highlighting the complexity of the gut ecosystem. Belenguer *et al.*[55] suggested that lactate was rapidly metabolized into acetate, butyrate and propionate by the intestinal microbiota at pH values of around 5.9. Nevertheless, at pH 5.2 the use of lactate is reduced, while its production is maintained, resulting in lactate accumulation. These data were similar to those found in our research, as values of acetate and butyrate are directly and inversely related to time and to the fermentation time, respectively.

The most important metabolites produced by bacteria during fermentation are, directly or indirectly, organic acids such as acetate (produced by *Bacteroides*, *Bifidobacterium*, *Lactobacillus*, *Enterobacteria etc.*), butyrate (*Firmicutes*), propionate (*Bacteroides*, *Prevotella* and *Clostridium*), lactate (*Lactobacillus*, *Bifidobacterium etc.*), succinate (*Lactobacillus*), together with hydrogen and CO_2.[54,60,61] Previous studies have reported that the production of acetate, propionate and butyrate mainly depends on carbohydrate fermentation. However, protein and amino acid fermentation also play an important role in this pool of SCFAs and also BCFAs.[62] SCFAs are mainly produced in the proximal colon in high concentrations (70–140 mM) and transported through the distal colon by the intestinal flow. SCFAs are absorbed and mainly used by colonocytes for their metabolic maintenance.[63] These bacterial metabolites have been associated with multiple biological activities, such as the regulation of energy homeostasis, anti-inflammatory activity[61,64] and satiety.[65,66] In addition, the supplementation of butyrate and acetate in a diet has shown to suppress weight gain in rodent models[67,68]. Propionate has also been associated with an inhibition of food intake in humans. Moreover, SCFA receptors, FFAR2 and FFAR3, located in the intestine are related to the expression of peptide YY and glucagon-like peptide 1 (GLP-1), two of the most important hormone mediators in satiety.[65,66] Another action point of SCFAs in satiety is the stimulation of the release of serotonin (5-hydroxytryptamine) through the activation of SCFA receptors, affecting colonic motility and the digestion time, leading to satiety regulation.[69] An antimicrobial function has also been associated with SCFAs causing a decrease in the colonic pH inhibiting the growth of some potential pathogens. Furthermore, it has been described that acetate promotes the release of reactive oxygen species (ROS), which are efficient bactericidal factors.[70] In relation to our results, Shen *et al.*[71] used meat as a fermentable substrate in a batch culture fermentation process, obtaining similar concentrations of butyric and i-butyric acid to our study. However, our data on acetate, caproate and valerate levels were higher, maybe caused by another whey substrate, such as lactose.

These fatty acids result from proteins and amino acid degradation and they could indicate a major degradation ability of microbiota, especially in OB, exposed to several whey proteins. The level of these BCFAs often comprises less than 10% of the total SCFA, potentially due to the low level of polypeptides and amino acids reaching the colon compared with non-digestible carbohydrates.[29] Their effects on the colonic epithelium have been poorly described, they could be probably involved in regulating ionic movements through the colonic epithelial layer.[72]

In relation to microbiota evolution, it is important to note that obesity is associated with an increase in the *Firmicutes*/*Bacteroidetes* ratio and a decrease in the diversity of microbiota due to both weight and diet.[7] The initial levels of *Firmicutes* in our study were consistent with this theory. This group of bacteria is associated with a high ability of harvest energy from the diet[7] and also related to low-grade intestinal inflammation, leading to the development of obesity and metabolic syndrome.[6] Our initial levels of *Bacteroides* counter this assertion. Classically, *Bacteroides* has been associated with NW, overall in the case of a fat- or carbohydrate-restricted diet (it is a bacteria responsive of calorie intake),[73] but Patil *et al.*[74] found an increase of this bacteria in OB Indian individuals. A recent meta-analysis did not find significant differences in many studies related to this and suggested that the PCR primer used, sample handling or extraction could be the cause of contradictory results.[75]

Lactobacilli (and also bifidobacteria) have a well-known probiotic effect on the gut microbiota.[76] However, several studies have associated *Lactobacillus* ingestion with obesity,[77–79] consistent with our results (initial lactobacilli were higher in OB than NW). Million *et al.*[10] confirmed this association when they studied the gut microbiota in OB and NW donors, finding that *Lactobacillus reuteri* was related to microbiota in OB, possibly because this bacterium improves the intestinal capacity to absorb nutrients and increase food conversion.[80] Nevertheless, other lactobacilli such as *L. paracasei* or *L. plantarum*; *Bifidobacterium* spp. (*B. animalis*) and *Methanobrevibacter smithii* are related to a normal body mass.[10]

Our study has demonstrated amply that liquid whey could be considered a high quality protein source, as well as a prebiotic because it contains approximately 5% lactose, which can be beneficial for increasing probiotic bacteria such as *Lactobacillus* and *Bifidobacterium*.[30] Moreover, this by-product from cheese production stabilizes or slightly decreases certain bacteria groups typical in OB people, such as *Firmicutes* and *Enterobacteriaceae*.

The high-quality proteins in whey composition should be taken into account in terms of their health benefits. Not only protein degradation happens during gastrointestinal digestion, but also proteolytic activity occurs in some groups of bacteria, such as *Bacteroides fragilis* and *Propionibacterium*, and other bacteria belonging to *Firmicutes* such as *Streptococcus*,

Clostridium, *Bacillus* and *Staphylococcus*.[51,52] Some studies have shown that *Bacteroides* have strong peptidase activity mainly at pH around 6.5.[81,82] Our data support these results because after 12 h of fermentation the pH lowered to less than 6.5. The reduction in *Bacteroides*, observed in the present study, could be due to the fact that from time 0 to 12 h of fermentation, the pH values decreased from 6.5 to approximately 4.9, reducing protease activity and, consequently, the stabilization of *Bacteroides*. Subsequently, the pH values continued to decrease until they reached 3.9, causing a reduction in these bacteria.

Results showed important changes in beneficial bacterial population such as bifidobacteria and lactobacilli for 48 h of fermentation of whey milk samples. In this regard, it should be noted that cow, sheep and the mixed whey showed the highest prebiotic effect at different times; however, goat whey seems not to exert an important positive effect on the growth of probiotics and the metabolite production in the gut. The high concentration of whey from cow (60%) could probably explain the similar trend observed in the mixture of the different types of whey milk. Increase in *Bifidobacterium* in both donors, OB and NW, correspond to a lower gas production and pH levels. Particularly high levels of bifidobacteria and low pH levels were found at 24 h of fermentation and it can be explained because *Bifidobacterium* could ferment lactose into acetate, lactate and pyruvic acid, which could reduce the intestinal pH. This fact could improve the intestinal health, preventing the growth of other pathogenic bacteria, such as *Salmonella* or *E. coli*.[31] Consequently, at 24 h the total gas production was not as pronounced as at 12 h because bifidobacteria are non-gas producing bacteria.

The level of *Lactobacillus* observed was very pronounced in OB donors. As we mentioned at the beginning of the Discussion, although some authors suggest that *Lactobacillus* (*L. reuteri*) may promote obesity,[10,77–79] this must be considered with caution because the mechanism by which this probiotic is associated with obesity still remains unclear. Further analysis to detect specific species of *Lactobacillus* must be carried out to determine better this association.

The increases in probiotic bacteria found in this study are consistent with several previous studies in which it was reported that milk protein and hydrolysed peptides increase *Bifidobacterium* growth.[83,84] Moreover, recent studies have demonstrated that whey protein intake increased the levels of *Lactobacillus* and *Bifidobacterium* in a rat model of colitis, possibly by an amino acid composition-mediated mechanism[27] or specifically increased the *Lactobacillus* population and decreased the *Clostridium* group in a mouse model.[28] Kobayashi *et al.*[85] demonstrated that liquid whey feeding for pigs enhanced probiotic bacteria, while opportunistic pathogen species disappeared. In addition, the ingestion of milk whey culture produces an active substance (1,4-dihydroxy-2-naphthoic acid (DHNA)), isolated from the whey culture with a dose-dependent bifidogenic effect, improving inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease.[26,86] And it is well known that GOS can also selectively promote *Bifidobacterium*[87–91] population. Different studies report that specific whey proteins have anti-microbial activity[22,24,25] through whey-derived peptides' formation by pepsin catalysed lactoferrin to lactoferricin.[23] In this regard, polypeptide fractions of α-lactalbumin (LTD1, LTD2 and LCD) have proven to have an effect on *E. coli*, *K. pneumoniae*, *S. aureus*, *etc.*[92] Moreover, milk proteins and peptides such as lactoferrin, lactoperoxidase and lysozyme, have provided a non-immune defence against microbial infections.[93] Regarding obesity, the prebiotic effect of whey is related to obesity prevention[94] by increasing *Bifidobacterium* population that could modulate low-grade intestinal inflammation in OB mice by stimulating GLP-2, which reduces cell permeability and therefore the entrance of LPS.

Conclusion

The present findings demonstrated that whey from milk of cow and sheep and also mixed, had a prebiotic effect on the gut microbiota of NW and OB donors during batch culture fermentation. These types of whey stimulated the growth of probiotic bacteria, demonstrating a marked bifidogenic effect in both groups of donors. Moreover, whey fermentation led to the production of a wide variety of SCFAs and BCFAs, especially in OB donors, which could be beneficial for colonic health. Supplementing diet with these types of whey can be a promising strategy to modulate the gut microbiota and to stimulate selectively the growth of probiotic bacteria. This could improve intestinal disorders and be an interesting and encouraging approach to the prevention of gut disorders, obesity and related diseases.

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