Whey protein fermentation as a predictor of satiety

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Obesity has increased in both, developed and developing countries. This is a reason for developing satiating ingredients. It has been shown that whey milk is effective in enhancing satiety and therefore, decreasing weight gain. Short chain fatty acids (SCFA) are metabolites produced by gut microbiota and closely related with satiety, since they are involved in releasing of anorexic peptides. The aim of this study was to evaluate the pH, gas variation and SCFA production after *in vitro* digestion and fecal fermentation of whey milk from several domestic mammalian species. We used faeces from normo-weight (NW) and obese (OB) volunteers. Statistically significant differences in SCFA profile between both types of donors make a clear distinction about the effect of whey fermentation on diversity of SCFA production, especially in cow, goat and mix whey. Gut microbiota of NW only were capable to produce high amount of acetate, meanwhile bacteria of OB produced high variety of SCFA even BCFA. These data highlight the role of whey protein in the colon and production of SCFA involved in satiety. Further studies about satiating effect of diet to enhance SCFA production with whey are neccesary.

Introduction

Obesity has increased dramatically during the past decades and has now reached epidemic proportions in both, developed and developing countries. Due to the obesogenic environment, it is necessary to develop new strategies focused on reducing the overweight and obesity through ingredients capable of increasing satiety. Considering different satiating efficacies of the macronutrients, protein is the most satiating one (1). It has been proved that whey protein (WP) has more satiating effect than casein, when both have been studied comparing food intake and postprandial metabolites and gut hormones release (2).

The satiating effect of whey is attributed to its protein fraction, galactooligossacharides (GOS) (3) and calcium contents (4), among others. It has been demonstrated that its protein content reduces food intake (5), stimulates gut hormone receptors, including CCK (6) and GLP-1 (7) and slows stomach emptying (8). Besides, the specific characteristic of WP can be affected by several factors such as animal species, breed, lactation period and feeding. Moreover, gut microbiota has taken an important role in the regulation of energy homeostasis and energy intake, existing obese and normal-weight profile bacteria (9). Whey protein could be fermented by colonic bacteria, producing short chain fatty acids (SCFA), branched chain fatty acids (BCFA) and ammonia (10). These SCFA are related closely with satiety, since SCFA may stimulate gut satiating hormones secretion via receptors of SCFA as FFAR2 and FFAR3 (11). In this study, we examine the effect of fermentation of whey from different mammalian species on SCFA, what could have implications on the design of new satiating ingredients based on kind of protein.

Material and methods

Whey samples

Four types of whey milk were used in this study: Friesian cow, Murciano-Granadina goat and mixture (80% cow and 20% sheep) were provided by Palancares Alimentación S.L. (Murcia, Spain) and Segureña sheep was provided by Quesos Vega Sotuélamos S.L. (Albacete, Spain). Each sample was lyophilized and stored away from light and humid for further assays.

Faecal samples

Human microbiota's samples were obtained from the faeces of 3 volunteers with normal-weight (NW) and from 3 obese (OB). Donors were healthy, without intestinal and/or metabolic disorders, not taken antibiotics, probiotics neither prebiotics in the last three months. Besides, volunteers were weight stable before entry on the experiment. Fecal samples were kept at 4ºC and processed within 1 hour of collection.

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

Simulated gastrointestinal digestion were performed using *in vitro* method being performed in three phases to simulate oral digestion, gastric digestion and intestinal digestion (12). 3 g of each sample of whey were homogenized with 3 mL of salivary saline solution (50:50 v/v) and maintained for 2 minutes in the water bath at 37 °C and 60 strokes per minute. Gastric phase was prepared with pepsin (Sigma-Aldrich) and was mixed with total volume from the oral phase and the pH was adjusted to 3. Then, sample was incubated in the same conditions as the oral phase but over a period of 2 hours. Intestinal phase intestinal solution was prepared and pancreatin (Sigma-Aldrich) and bile salts (Sigma-Aldrich). Then, intestinal solution was mixed with the volume from the gastric phase, adjusting the pH to 7. After that, sample was incubated for 2 hours in the water bath similar than before. Finished the whole process, sample obtained was frozen at -80 °C to stop enzymatic activity.

Faecal batch culture fermentation

The fermentation profile of digested whey, inulin and glucose were determined in duplicated, using anaerobic faecal fermentations. Stabilization of faeces were carried mixing 3 grams of faecal sample and 27 mL of PBS, achieving a dilution 1:10 (w:v). Fecal samples were stabilized injecting 15 mL of homogenized fecal mixture into previously prepared bottles with of minimal basal medium (MBM) and anaerobic conditions, as described by (13) and (14), an incubated at 37º C with agitation for 4 h. Finally, MBM was poured onto wheat on serum bottles (100 mL), previously autoclaved and containing the digested whey at final concentration of 1%. Then and after fecal slurry stabilization, bottles were inoculated with 7 mL of faecal slurry. All samples were incubated in water shaking bath set at 37º C and fermented for 48 hours; pH values and gas production were measured at 0, 12, 24 and 48 h. The volume of gas production generated was assessed during all fermentation process (15) by inserting a sterile needle attached to a pressure transmitter . Aliquots of 8 mL were taken after 0, 12, 24 and 48 h of incubation per bottle taking for duplicated SCFA analysis. Aliquots were then centrifuged at 12000 x g for 15 minutes and stored at -80º C.

Analysis of short chain fatty acid (SCFA)

Short chain fatty acid production was analyzed by gas chromatography. An internal standard of 2-Ethylbutyric acid was prepared with methanol. Two calibration curves were

done, one with different concentrations of a volatile acid standard mix (VASM) and another with acetic acid. The chromatographic conditions were: constant pressure to elution, and 2.2 mL/min of gas flow. The injector and detector (FID) temperature was set at 280º C. Oven was programmed maintaining 185º C for 3 min.

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 homocedasticity were analyzed of variances by the Shapiro-Wilk test and Levene respectively, setting a level of significance ($p < 0.05$).

Results and discussion

Before explain data, we must consider that many inherent differences exist in the microbiota of donors. Also, whey composition from different species can be reflected in their fermentation products. Moreover, few studies about fermentation of whey are now available and none studying differences on fecal fermentations of different types of whey on the SCFA production from studying satiety in humans.

The pH of all samples decreased from 6.5 to 4 after 48h of fermentation ($p<0.05$). We must to highlight an important decrease between 0 to 12 h. No differences were found when compare whey or donors. This fall of pH may reflect SCFA production through fermented process. In this regards, GOS could act as a prebiotic on colonic bacteria leading to produce mainly lactate and acetate by *Bifidobacterium* and *Lactobacillus* (16). These compounds decrease the pH, inhibiting the activity of other bacteria that metabolize lactic acid to produce propionic acid (17).

Regarding gas production, we found differences ($p<0.05$) at different times, obtaining the highest pressure increase at 12 h. Besides, NW donor shown the highest amount of gas, specially cow whey samples.The lowest gas production were recorded with inulin followed by glucose in both donors. An inverse relation between presence of *Bifidobacterium* and gas production has been reported (18). During first 12 hours of fermentation a big gas increase happened, maybe because bifidobacteria grow lower than other proteolytic bacteria such as *Clostridium* and *Streptococcus*, probably stimulated by whey.

Saccharolysis and proteolysis are the main fermentations carried out by colonic bacteria. The major ends products of bacteria fermentation are organic acids such as acetate, butyrate, propionate, lactate, succinate together with hydrogen and CO_2 (19). Production of acetate, propionate and butyrate depends, mainly, of carbohydrate fermentation. Besides, protein and amino acid fermentation have an important role in this pool of SCFA through proteolytic bacteria, producing SCFA and BCFA such as i-butyrate, i-valerate and 2-methylbutyrate, ammonia, among others (20).

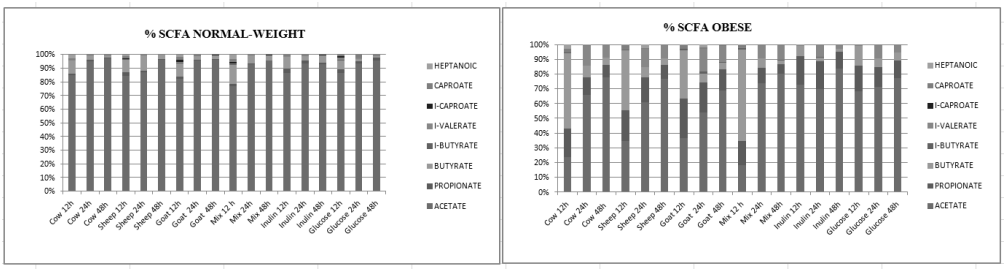

Figure 1. Molar concentration percentage (%mM) of acetate, propionate, butyrate and minor SCFA produced during fermentation at 12, 24 and 48 h.

The concentration percentage (Figure 1) of acetate, propionate, butyrate and minor SCFA (i-butyrate, i-valeric, valerate, i-caproate, caproate and heptanoic acids) produced during *in vitro* fermentation. The concentration of total SCFA increased significantly upon whey substrates fermentation, showing a positive correlation between time of fermentation and amount of SCFA.

Total concentration of SCFA in whey fermented was similar in both donors. A high amount of SCFA in cow, sheep and mix whey samples were found at time 48. Acetate and propionate showed an increased tendency along fermentation time. Significant increase ($p<0.05$) was observed in concentration of acetate in whey samples, specially cow, sheep and mix whey, of NW respect OB. The highest values were detected after 48 h. Furthermore, OB donor yield the highest amount of butyrate and propionate in all samples, being much more higher than NW. Propionate values were specially higher in case of mixture and sheep whey, at time 48 and 24 h, respectively. In relation with butyrate, OB donor presented a significant ($p<0.05$) increase at time 12 h in all samples of whey, highlighted in cow and mix whey. Nevertheless, butyrate levels began to fall from 12 h fermentation in OB donors. NW donor presented minimal amount of propionate and modest levels of butyrate, especially in sheep whey. We found significant increased of i-valerate in OB, with high values of at time 24 and 48 h in all whey samples, especially for mix whey. In spite of only found significant level of i-valeric acid, it is characteristic of protein and amino acid degradation and it could be reflex of a major degradation ability of microbiota of OB exposed to several whey proteins. Shen *et al*., (2010) using as substrate meat showed, similar concentrations of butyric and i-butyric acid that those found in our study, however our data of acetic, caproic and valeric were higher. (21). In relation to the effects of BCFA on the colonic epithelium, have been poorly described and probably, they may be may be involvement in regulating ionic movements through the colonic epithelial layer (22). Rycroft *et al*., (2001) showed that GOS was fermented to lactate and acetate achieving the highest values at time 5 and 24 h; meanwhile, propionate and butyrate decreased. In addition, Belenguer *et al.,* (2007) suggested that lactate was rapidly converted to acetate, butyrate, and propionate by the human intestinal microbiota, at pH values were low as 5.9, but at pH 5.2 reduced utilization occurs while production is maintained, resulting in lactate accumulation. This data are according with us, since values of acetate are directly related with time to fermentation and butyrate, inversely related in OB. Proposal explanation at this phenomenon could be that in our study we not only consider GOS as a substrate of fermentation, since protein present in whey could be fermented and thus stimulating propionic bacteria producer.

Conclusions

Whey samples fermented by faeces of obese and normal-weight present different SCFA profile. Interestingly, obese donor showed much more variety of SCFA producer, specially, with cow, sheep and mix whey. These data could explain how different types of whwy may be able to modulate the intestinal microbiota can take place to a desirable profile of fatty acids related with satiety.

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