The SCFAs Production of Syntrophic Culture of L. johnsonii SZ-YL and A. Muciniphila in Different Macrobutrients

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Abstract. The gut microbiota is a complex ecological system that influences numerous host physiology, such as obesity, appetite, immunity and drug metabolism. As a crucial component in intestine, short chain fatty acids (SCFAs) can modulate host metabolism and disease physiology via receptor, such as GPCRs. The system of knowledge of the host gut microbiota has been greatly expanded. Diets alterations can rapidly modify gut microbial composition and affect SCFAs production. Current knowledge of SCFAs production is mainly based on isolates or intestinal environment, but these may not reflect the effects of bacterial interaction on SCFAs generation. In these studies, the potential effects and SCFAs production of in a syntrophic coculture of *L. johnsonii* and *A. muciniphila* was investigated in different medium. Cocultures produced higher acetate and propionate in glucose and inulin medium. In oleic acid addition medium, the growth of *L. johnsonii* were promoted and the acetate production were promoted. These results indicate that appropriate diet proportion is necessary for beneficial bacteria growth to affect host health. Moreover, unsaturated fatty acid promotes the growth of Lactobacillus instead of *A. muciniphila*. Our results indicated that the changes of diet proportion might be potential method for beneficial bacteria growth.

Keywords: Lactobacillus; A. Muciniphila; SCFAs.

1. Introduction

The gut microbiota is a collection of microorganisms (all prokaryotes, eukaryotes and viruses) present in the intestinal environment [1]. The gut microbiota colonized on colon contains as many as 1 tillion bacterial cells and accounts for 70% of total microbes in the huamn body [2]. The variability and complexities of gut microbiota is closely associated with normal host physiology and diseases, from inflammatory bowel diseases (IBD) to chronic autoimmune inflammatory diseases [3]. If there is growing evidence in life that dietary or non-dietary lifestyles are modulating the makeup of gut microbiota to affect host metabolism [4]. For example, stress can affect colonic motility activity and alter microbial composition, and it is notable that as stress increases, the obvious effect is a decline in the number of Lactobacillus in the gut motility [5]. Among the lifestyles, diet is one of the most potent influences in altering bacterial composition and host diseases, especially obesity and type II diabetes [6]. High fat diet, the critical factor of obesity, induce the increase of circulating levels of bacterial lipopolysaccharide (LPS) and abundance of Firmicutes [7]. The contribution of gut microbiota in host health after diet changes might be due to the micro molecule fermented from gut bacteria, such as short chain fatty acid (SCFA) [2].

As the main fermentation component of dietary fiber, acetate, propionate and butyrate are the main SCFA constituents, of which the molar mass ratio is about 60:20:20 [8]. SCFAs are involved in the energy supply of intestinal epithelial cells, regulation of intestinal immunity, anti-colon tumor, and have therapeutic [9]. Increasing interest in the role of SCFAs is their effect on host metabolism (glucose and lipid metabolism) [10]. Among SCFAs, acetate, produced by most specific bacteria, plays a role in control appetite and satiety, accelerates lipid oxidation and alters inflammation levels [11, 12]. Propionate is produced from several substrates and can directly reduce appetite and food intake. In human model, intake of propionate has been shown to promote lipid oxidation. In addition, the function of propionate to inhibit cell proliferation is suggested to its role as G protein-coupled receptor (GPCR) agonist [13]. In animals, oral administration of butyrate has been shown to change appetite and change lipid metabolism via nervous system [14]. In high BMI males, colonic SCFA supplementation appears to change energy consumption rate [15].

The function of many single strains for SCFA production of intestinal bacteria has been demonstrated, for example, Lactobacilli, which ferment carbohydrates into acetate and lactic acid to affect host health [16]. Some species of Lactobacillus are well-studied probiotic that colonizes a large number of mammals [17]. As research progressed several beneficial effects of *L. reuteri* were discovered, such as development of regulatory T cells and antibacterial substances production [18]. It is worth mentioning another intestinal bacterium, *Akkermansia* is an oval-shaped gram-negative bacterium that is a resident of the human intestine, accounting for 3-5% of the human microbial community [19]. *Akkermansia muciniphila* can successfully colonize the intestine and degrade human mucin [20]. The abundance of *Akkermansia* bacteria was reduced with diet-induced obesity in mice and type 2 diabetes in human by altering adipose tissue metabolism and intestinal permeability [21]. *L. pentosus* was reported that can interact with *Akkermansia* to increase its abundance to regualte immunity in intestine [22].

The development of culture-based and sequencing technologies has greatly expended the knowledge of the interaction of gut bacteria with health and disease [23]. Despite the diets, especially macronutrients, which has a major role in altering gut microbiota or single bacterium and their activity, there are many questions that need to answer. The impacts of dietary macronutrients on the SCFA production from several probiotics are less well defined. Here, the impact of macronutrients, including fat, protein and fiber, on the co-culture of *Akkermansia* and *Lactobacillus* is described. It elucidated that the underlying mechanism of how macronutrients affect host healthy via bacteria interaction.

2. Materials and Methods

2.1 Media and Strain

The modified BHI media consists of classical BHI medium with acetic acid (1.9 mL), propionic acid (0.7 mL), iso-butyric acid (0.09 mL), n-valeric acid (0.1 mL), iso-valeric acid (0.1 mL), mucin (1‰ w/v), and distilled water (1000 mL). In protein-rich media, the final concentration of protein was reached to 11 g/L. In glucose-rich media and inulin-rich media, the content of glucose and inulin are both 4 g/L. In fatty acid media, the concentration of oleic acid was 200 mM. *Akkermansia muciniphila* was purchased from American Type Culture Collection (ATCC).

2.2 Isolation of Lactobacillus

The feces collected from mice (C57BL/6) were immediately transported to the anaerobic incubator. The feces were homogenized in 0.9% NaCl containing 0.1% cysteine. Diluted the suspension, and then spread on agar plate with modified BHI media. Incubated the bacterium in anaerobic incubator at 37℃ for 2-5 days. Single colonies were streaked on new plates three times for single clones.

2.3 16S rRNA Gene Sequencing and Phylogeny

The resulting PCR product from 16S rRNA gene was sequenced by RuiBiotech. Sequence similarity were calculated between using NCBI blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment was conducted and the phylogenetic trees were reconstructed by neighbor-joining using MEGA software.

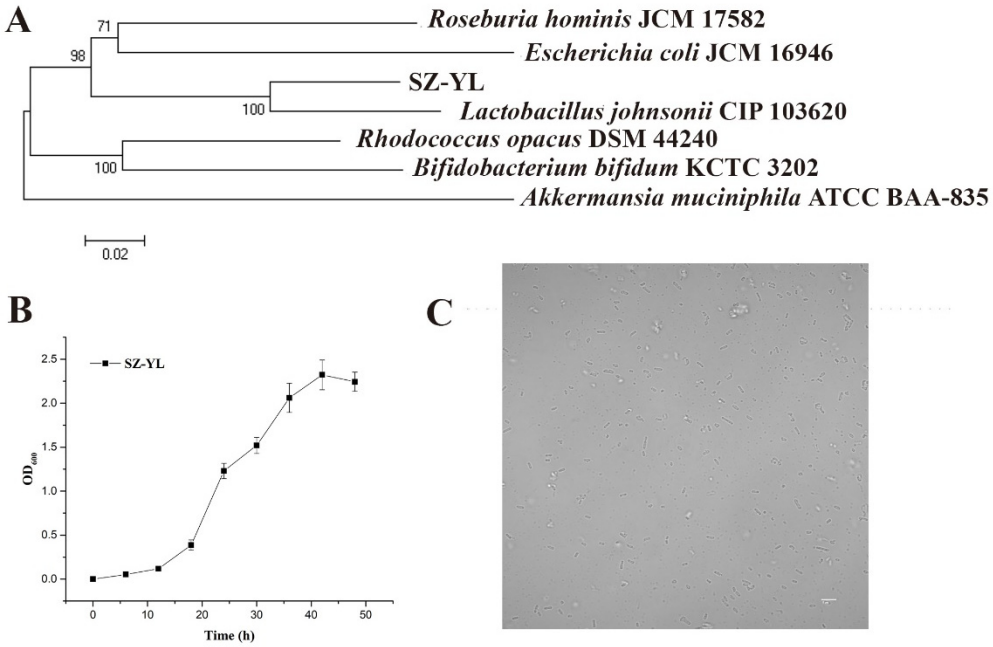
2.4 Fluorescent in Situ Hybridization (FISH)

Probe was synthesized with a FAME reactive fluorescent dye at the 5’ end (RuiBiotech). Species-specific FISH probes were designed in probeBase (https://probebase.csb.univie.ac.at/). The MUC-1437 was used to detect virtually *Akkermansia muciniphila.*

2.5 SCFA Measurement

Collected 2 ml medium and centrifuged at 20,000g for 5 min. The supernatant was added deproteinizing solution (25 g metaphosphoric acid and 0.217 mL 2-ethyl butyric acid per 100 ml). Incubated the solution in ice for 1 hour and then centrifuged at 20,000*g* for 10 min. Collected the supernatants and then filtrated by 0.22μm membrane filters for SCFA analysis. SCFA were analyzed using gas chromatography (SP-3420A, Beifenrili Analyzer Associates, Beijing, China) with a capillary column (AT-FFAP: 30 m × 0.32 mm × 0.5 μm) as previous reports [24].

3. Results and Discussion



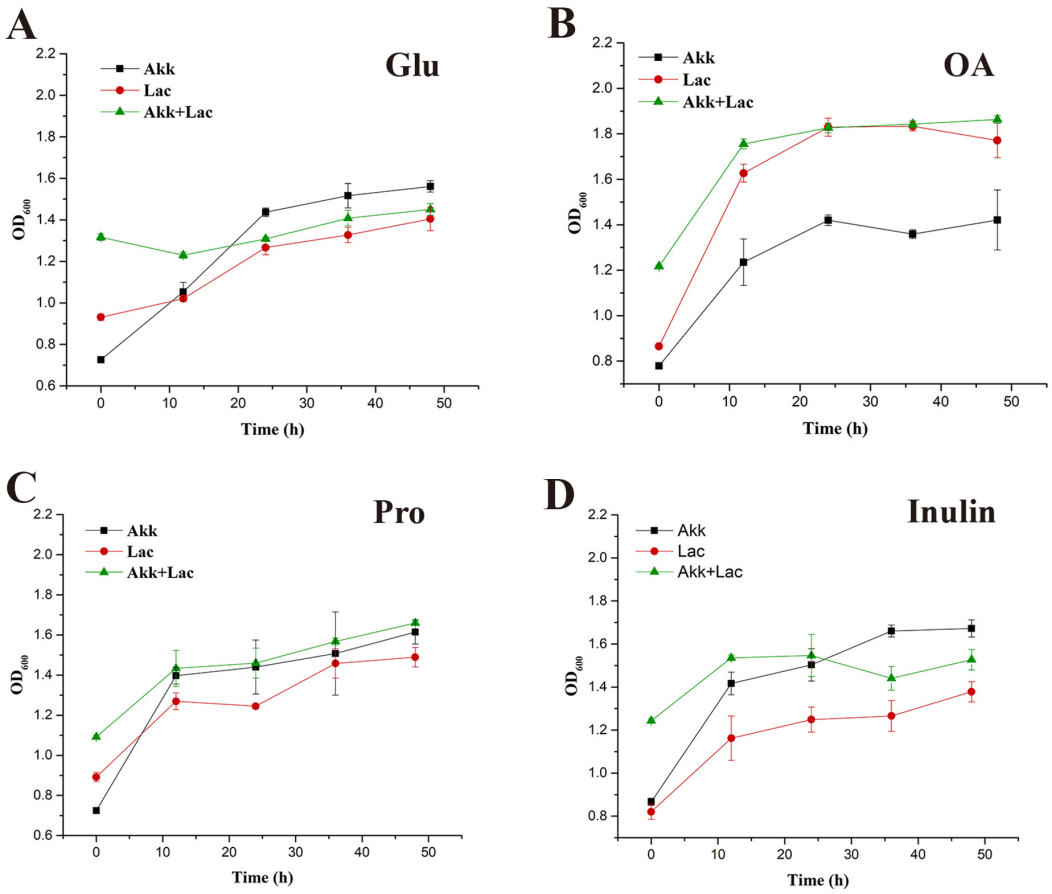
(A) Phylogenetic tree (16S rRNA gene sequence) showing the relationship of the strain SZ-YL to members of the *Lactobacillus johnsonii*. (B) The growth curve of the strain SZ-YL. (C) Confocal image of the strain SZ-YL.

Figure 1. Isolation of *Lactobacillus johnsonii* from mouse feces

3.1 Isolation and Characterization of Lactobacillus Johnsonii

For the isolation of gut bacteria, the microbiota sample collected from the mouse feces and diluted it to a concentration of 10^{-5}-10^{-7} (v/v) on modified BHI medium. One colony from plate was analyzed for its 16S rRNA gene sequence. Sequences of the strain SZ-YL has more than 99% identity with *Lactobacillus johnsonii*. The phylogenetic analysis is shown in Figure 1A. The strain SZ-YL, which are similar to *L. johnosonii*, is Gram-positive strains, and is fermentative under anaerobic conditions. In Figure 1B, SZ-YL, because of its absolute similarity to *L. johnosonii*, the growth curve shows that the plateau period occurs from approximately 25h-33h, when the growth of strain SZ-YL is relatively slow. During the previous period (about 20h-25h), the bacterial growth was relatively rapid and after the plateau period, the bacterial growth rate also showed a significant increase (see about 33h-40h). The duration of the plateau period is approximately five to six hours. It is worth noting that the inflection point occurs between 40h and 45h, when the OD600 starts to show a decrease, from a value of about 2.25. At this time the bacterial colonies have passed the interval between 35 and 45h, which is also a specific plateau period, where the bacterial colonies show a slowdown in growth and start to decrease in number after the end. The plateau period before the

reduction lasted mainly five to six hours. As shown in the confocal image in Figure 1C, we can see that the slender strips of rod-shaped bacteria, *L. johnosonii*, are mainly between about 5 μm in length.



The growth curve of *L. johnsonii*, *A. muciniphila* and co-culture (*L. johnsonii* and *A. muciniphila*) in (A) glucose-rich medium, (B) fatty acid-rich medium, (C) protein-rich medium and (D) inulin-rich medium

Figure 2. The effects of different macronutrient on the growth of *L. johnsonii* and *A. muciniphila*

3.2 The Effect of Different Macronutrient on Bacterial Growth Curve

Different bacterial species have preferences for different macronutrients, and with reference to the growth curves we were able to decipher the preference of different species for substances. In order to study the contribution of different energy-yielding macronutrients on gut bacteria community, we co-cultured *L. johnsonii* and *A. muciniphila* to elucidate the effect of macronutrient on bacterial metabolic. Referring to the growth curve in Figure 2, in the case of glucose-rich medium, *A. muciniphila* showed a very significant growth around 0-25h, and the growth of *L. johnsonii* was going on at the same time. However, when both bacteria reached around 25 h, their growth showed a certain degree of decreasing rate. This indicates the relative ability of glucose to promote the growth of both bacteria. Moreover, there is same pattern of growth about bacterial co-culture and single bacterial culture in glucose-rich medium. However, the final bacterial density of bacterial co-culture is slightly lower than the density of single cultivation of *A. muciniphila*. In the OA-rich environment, the growth curve represents a significant impression of OA on the growth of the strain SZ-YL (showing a very strong promotion effect). In fact, *L. johnsonii* showed a stronger affinity for OA at this point, and the growth of *A. muciniphila* was not as fast as its growth. In the protein-rich environment, the green growth curve shows that the protein has a slightly weaker but still significant effect on the growth of

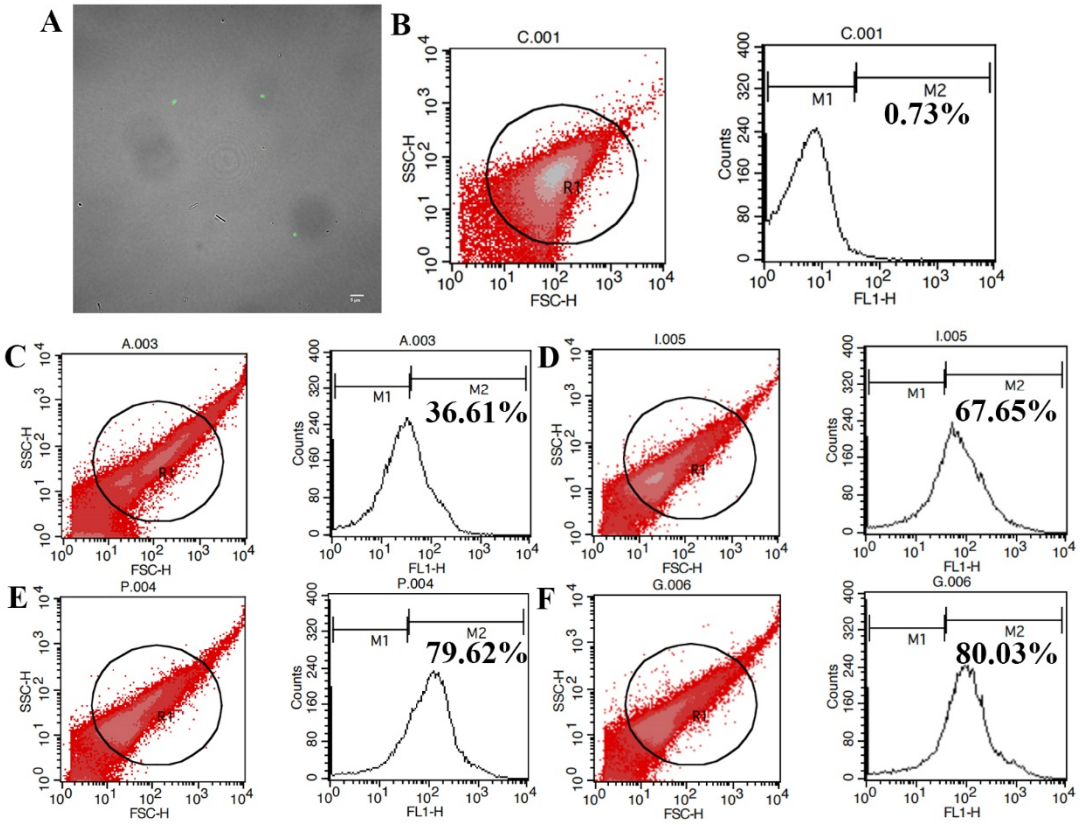
the bacteria compared to OA. It can be seen that *A. muciniphila* is more inclined to protein than *L. johnsonii*. In the inulin environment, *L. johnsonii* still did not show a stronger tendency than *A. muciniphila*. After increasing with time, it was clear that inulin could increase the density of *A. muciniphila*.

In human, metabolic endotoxemia could be induced after high carbohydrate [25]. Very-low carbohydrate diets are used as a method for weight loss [26]. However, some studies reported that low carbohydrate diets may have an adverse impact on the gastrointestinal health by affecting gut microbiota composition, such as the decrease of counts of bifidobacterial [27]. In our study, glucose could both promote the growth of single culture and co-culture of *A. muciniphila.* It indicated that appropriate amount of carbohydrate is needy for intestinal health. High fat diet (HFD) has contributed to the obesity epidemic [28]. As a healthier sources of fat for replacement of saturated fat, OA did not promote the growth of *A. muciniphila.* Instead, OA appears to increase the density of the strain *L. johnsonii* SZ-YL. These results mean that the effects of OA on host health could contribute to Lactobacillus, instead of *A. muciniphila.* Generally, ketogenic diet (high protein and low carbohydrate diets) has become popularity for weight loss and type 2 diabetes management [29]. In protein-rich media, co-culture has higher density of bacteria. The result is in line with the previous reported that protein concentration is not a major driver shaping the gut microbiota to affect host health [30]. It indicated that protein content is a main factor for gut bacterial growth. As one of dietary fiber, inulin could affect host health via regulated *A. muciniphila* [31]. Moreover, dietary fiber improves human health by promoting beneficial microbes' growth, such as Lactobacills [32]. In our study, inulin could both increase the bacterial density and shortened the time for reaching plateau period.

3.3 Fluorescent in Situ Hybridization of Bacterial Composition on Different Media

In order to explore the changes of bacterial composition in different nutrients, fluorescent *in situ* hybridization (FISH) was used to detected the ratio of *A. muciniphila* in co-culture media. In Fig 3A, we can prove the accuracy of the experiment because the bacterial species showing fluorescence is *A. muciniphila* and the one showing rods is *L. johnsonii* SZ-YL, demonstrating that the fluorescence in situ hybridization technique of the bacterial composition of the medium can help to distinguish between different species of bacteria and their numbers and density. Compared to initial ratio of *L. johnsonii* and *A. muciniphila* (about 1:1), there is a very clear indication of the increase in occupancy of *A. muciniphila* in the presence of high glucose. In this case, the percentage of M2 increases to 80.03%. Conversely, the presence of OA has opposite effect on the growth of *A. muciniphila*. And the M2 value was decreased to 36.61%. As shown in Figure 3.E, the number of bacteria also showed an increase when in a high protein environment. In the case of high protein, the value of M2 reached 79.62%. In Fig 3D, the inulin, one of dietary fibers, has an increasing effect on the *A. muciniphila*. As shown in the figure, the percentage of M2 grows to 67.65%.

The different bacteria have different substrate preferences [33]. And the hardwired differences in bacterial preferences for different macronutrients are helpful in their stable coexistence in complex environment [34]. The results of the ratio of *A. muciniphila* in glucose-rich environment indicate that the increase in intake of sugar or protein will have more significant effect on *A. muciniphila*. However, in previous studies, a low carbohydrate diet, high protein diet, induce the gut microbial changes with high abundance of *A. muciniphila* in mice [35]. The increase of density of *A. muciniphila* in glucose-rich media may be due to lack of competition from some bacteria which has preference of glucose. Referring to Fig 3C, the benefits of unsaturated fatty acid on host health may be partly because of its promotion on the growth of *Lactobacillus.* Moreover, the dietary fiber might be to promote host health through the growth of *A. muciniphila.*



(A) Confocal image of Fluorescent *in situ* hybridization about *A. muciniphila.* (B) Control group. (C) OA-rich group. (D) Inulin-rich group. (E) Protein-rich group. (F) Glucose-rich group

Figure 3. The change of bacterial composition in different media

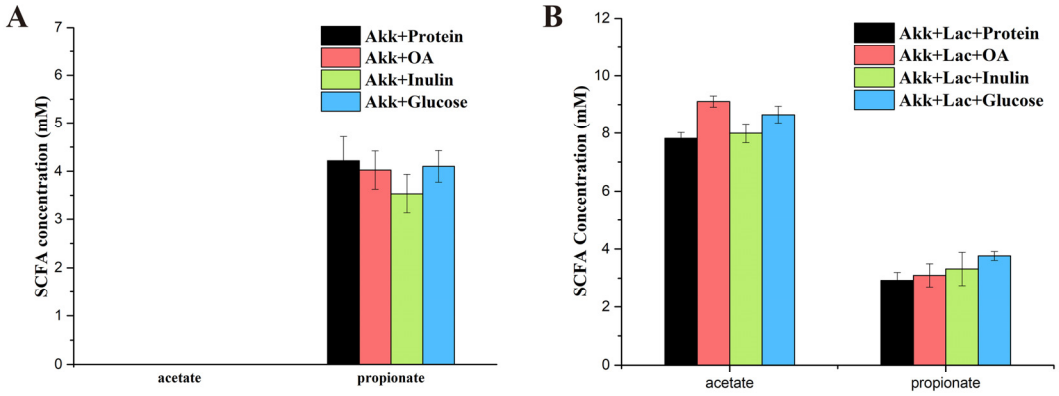


Figure 4. The SCFAs production of (A) *A. muciniphila* and (B) co-cultures

3.4 The Effects of Different Macronutrient on the Production of SCFA from Co-culture L. Johnsonii and A. Muciniphila

As key bacterial metabolites, short-chain fatty acids (SCFAs) can activate G-coupled receptors, involve in epigenetic and serve as energy source to affect host health [9]. In order to elucidate the potential effect of *L. johnsonii* and *A. muciniphila* after substrate induction, we measured the changes of SCFAs in different medium. As shown in Fig 4A, the ability of SCFAs production from *A. muciniphila* was measured in different medium. In protein-rich and glucose-rich medium, *A. muciniphila* can produce higher concentration of propionate. Moreover, *A. muciniphila* slightly convert OA to produce SCFAs compared to in inulin medium. Fig 4B shows the variation of SCFAs in co-culture of *A. muciniphila* and *L. johnsonii* under different cultures, where the production of SCFA was mainly derived from the bacterial co-fermentation. As shown in the Fig 4B, the content of acetate was the highest and around 9 mM in the high OA environment. Under glucose-rich culture, bacteria can produce different SCFAs well, especially propionate. Similarly, in the protein-rich case, not only the acetate production was greatly reduced, but also the propionate was decrease. In inulin environment, SCFAs production were not as much as except.

The core functions of *A. muciniphila* physiology due to mucin degradation are production of propionate after mucin degradation [36]. As shown in Fig 4A, the value of propionate was around 4 mM, bacteria clearly grow well in protein environments due to *A. muciniphila* prefer proteins. In the culture environment of inulin, the bacterial production of the lowest number of acetates might be due to the lack of enzyme for inulin degradation in *A. muciniphila.* At the same time, the number of propionates in glucose-rich culture proved that *A. muciniphila* could use monosaccharide and OA rather dietary fiber to produce propionate. Moreover, the bacteria lack the pathway to produce acetate. As shown in Fig 4B, the growth of *Lactobacliius* leads to a certain degree of limitation in the growth of *A. muciniphila* to limitate its ability to produce SCFAs. In all four environments, butyrate cannot be detected which can prove that both bacteria lack butyrate generation pathway. When comparing the production of SCFAs in different medium, it is clear that glucose is easier to utilize to produce SCFA. Unsaturated fatty acid can promote the growth of *L. johnsonii* SZ-YL and then produce more acetate. Due to the lack of genes of inulin degradation, the concentrations of propionate are similar in inulin culture even in co-culture. Protein can also promote the growth of Lactobacillus, which results in the acetate production. All results indicate that the ratio of different diet is more important than the intake of single nutrients. In today's society, with the constant lack of attention to the balance of food intake, the environment of a high-protein diet certainly makes us lack the attention to dietary fiber intake. Balanced diets can improve intestinal health via beneficial bacteria growth and SCFAs production.

4. Conclusion

The phylogenetic analysis of the isolate of anaerobic bacteria was shown to cluster with *Lactobacillus johnsonii*. Fatty acids have been reported that dietary fatty acids are converted to biological activities compounds by enzymes from host or gut bacteria to affect host health. As an unsaturated fatty acid, OA could promote the growth of *L. johnsonii* to produce acetate and has no effect on the growth of *A. muciniphila*. These results partly proved that unsaturated fatty acid might play their role on host via *Lactobacillus*. Although proteins promote the growth of *A. muciniphila* to produce propionate, *Lactobacillus* compete with them on protein to generate more acetate. Glucose is a common monosaccharide to be used by both bacteria. Therefore, concentrations of acetate and propionate in glucose culture are higher than others. Moreover, due to the lack of efficient enzymes for dietary fiber utilization, co-culture produces lower concentration of acetate and propionate. In conclusion, the appropriate dietary structure is more important than simple diet. If the simply diet is used for modulating host health, more studies about co-culture of gut bacteria are necessary.

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