



# Effect of *Lactobacillus plantarum* DAD-13 and Fructo-oligosaccharides on Short-Chain Fatty Acid Profile and Nutritional Status in Indonesian Stunting Children

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## Abstract

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**BACKGROUND:** Chronic gut inflammation is a generalized disturbance of small intestine structure and function is likely to play a large role in the incidence of stunting. It will be disturbances the absorption of nutrients, therefore, it can indirectly reduce on nutritional status.

**AIM:** The aim of this study is to examine the effect of *Lactobacillus plantarum* DAD-13 and fructooligosaccharide on short-chain fatty acid (SCFA) profile and nutritional status in Indonesian stunting children.

**METHODS:** The study design was used double-blind randomized placebo-controlled trial, 39 stunting children under five received daily oral supplementations of *L. plantarum* DAD-13  $1 \times 10^{10}$  cfu and fructooligosaccharide 700 mg (symbiotic group) or placebo group for 90 days. SCFA profile was analyzed using gas chromatography and nutritional status was assessed by WAZ, HAZ, and WHZ.

**RESULTS:** The result shows in symbiotic and control group, the mean age was  $26 \pm 8.34$  and  $29 \pm 5.78$ , and the mean weight was  $8.5 \pm 0.94$  kg and  $9.0 \pm 0.82$  kg, while the mean height was  $78.96 \pm 5.4$  cm and  $80.9 \pm 4.55$  cm, respectively. Concentrations of acetate, propionate, and butyrate in the symbiotic group after consumption were  $17.10 \pm 2.97$ ,  $7.70 \pm 2.05$ , and  $7.47 \pm 1.76$  while in placebo group  $12.44 \pm 3.61$ ,  $5.20 \pm 1.66$ , and  $6.12 \pm 1.16$ , respectively. There was a significant difference in the mean SCFA concentration between the symbiotic and placebo groups ( $p < 0.05$ ), where the SCFA concentration in the symbiotic group was significantly higher than the placebo group. Nutritional status (WAZ, HAZ, and WHZ) was observed significantly in symbiotic group ( $p < 0.05$ ), only on WHZ has cutoff point  $> -2SD$  after the intervention, while WAZ and HAZ  $< -2SD$ .

**CONCLUSIONS:** *L. plantarum* DAD-13 and fructooligosaccharide 90 days supplementation have increase acetate, butyrate, and propionate that are important fuels for intestinal epithelial cells that can play an important role in the maintenance of health.

## Introduction

Stunting is one of the problems on children under 5 years in Indonesia and other developing countries [1]. Stunting is defined by a low height-for-age z (HAZ) score ( $< -2SD$ ) according to the World Health Organization Child Growth Standards. Stunting is associated with insufficient nutritional intake to support the rapid growth and development of infants and children, followed by recurrent infectious diseases that occur early in life [2], [3]. Based on basic health research in 2018, the incidence of stunting in Indonesia is 30.8%. This number is still very far from the target set by the WHO, which is 20% [1]. Therefore, this is a problem that must be given more attention as stunting (linear growth failure) resulted in irreversible impact [4].

Recently, evidence suggested that a chronic syndrome which causes inflammation of on small

intestine, known as pediatric environmental enteropathy (PEE), is likely to play a large role in the incidence of stunting. Stunting child gut has an abundance of enteric pathogens, namely, Enterobacteriaceae and other pathogenic bacteria. Consequently, the beneficial microbes level will be low, which are known to cause inflammation on gut [5]. There will be a low level of short-chain fatty acid (SCFA) especially acetate, propionate, and butyrate and elevation in stools pH, if there is an imbalance or loss of bacterial diversity in the human digestive tract or dysbiosis. If this happens, there will be disturbance the absorption of nutrients, both macro and micronutrients; thus, it is expected to indirectly decrease nutritional status (WAZ, HAZ, or WHZ) on children with stunting [6]. Stunted children with dysbiosis cannot recover only by a nutritious diet alone, but they also need probiotic, prebiotic, and symbiotic to modulate the microbiota composition especially microbiota containing potentially beneficial for human health [7], [8], [9], [10].

Probiotics are live microorganisms that can provide good or health effects on other organisms/their hosts. Prebiotics are non-viable food components, with a beneficial effect on the host and are associated with microbiota modulation. Aside from its ability to enhance probiotic bacterial species, prebiotic shown to have an effect on improving physiological health [11]. Symbiotic is products that contain probiotic and prebiotic agents. The combination is supposed to enhance the survival of the probiotic bacteria through the upper gastrointestinal tract, improve implantation of prebiotic in colon, and stimulate the growth and/or activities of both the exogenously provided probiotic strain(s). The advantage of combining probiotic and prebiotic mainly comes from their abilities to increase the numbers of beneficial organism in colon, reduce the numbers of potentially pathogenic microorganisms, and stimulate SCFA production [12].

SCFA derived from bacteria-dependent hydrolysis of plant materials, such as celluloses, fiber, starches, and sugars [13], [14]. However, when plant materials supply decreases, some bacterial species can switch to amino acid and protein fermentation as an alternative energy source, which also contributing to SCFA. SCFAs are important fuels for intestinal epithelial cells (IECs) and to regulate IECs function through different mechanisms. The example of IEC function is the modulation of proliferation and cell metabolism, differentiation which influences gut motility and strengthens intestinal barrier functions and also plays a role in weight regulation [14], [15]. A healthy gut microbiota gives an important role to human metabolism by contributing enzymes that are not encoded by the human genome, for example, the breakdown of polysaccharides, polyphenols, and synthesis of vitamins, which are essential for human health [10], [16], [17]. It performs various protective, structural, and metabolism bowel function; especially the production of SCFAs. SCFA has three predominant forms of fatty acids, namely, acetate, propionate, and butyrate; each has a beneficial role for human health, such as improving the gut profile of the microbiota, nutrient intake, and improving nutritional status [14]. SCFAs act as source of nutrition and proliferation for IECs, development mucosal immune system, production of certain vitamins, promote mineral absorption, affect metabolism rate, and appetite, induction of host genes for nutrition absorption: All of which are essential for optimal nutrition absorption [18].

## Methods

### Study design

A randomized, double-blind, and placebo-controlled study was performed using symbiotic powder

and skim milk (placebo). The clinical study period lasted 90 days from January to April 2020 at Rumah Pemulihan Gizi in Yogyakarta, Indonesia. Children under five with stunting were recruited in this study. The sample determination of stunting children under five was carried out by anthropometric screening for height-for-age (HAZ). Children with HAZ  $<-2$  SD were chosen as research subjects. The sample size was calculated using hypothesis testing for differences in two proportions between two independence groups. The minimal number of subjects calculated, with 95% confidence interval, power of 80%–10% drop out into account. Subjects were randomly divided into two groups: 19 participants in symbiotic group and 20 participants in placebo group. The inclusion criteria are children aged 12–59 months with stunting and did not take probiotics a month before the study took place. During the study period, symbiotic powder group consumed 1 g of symbiotic powder once a day, while placebo group consumed 1 g of skim milk each day.

### Research products

The study product was a symbiotic powder containing the indigenous probiotic strain of *Lactobacillus plantarum* DAD-13  $1 \times 10^{10}$  CFU and 700 mg of fructooligosaccharides (FOS). The symbiotic powder was consumed 1 g/day. The indigenous probiotic strain was deposited in ampoules at the Food and Nutrition Culture Collection (FNCC), Center for Food and Nutrition Studies, Universitas Gadjah Mada while the FOS was obtained from PT Beneo GmbH. The placebo group consumed a commercial skim milk as much as 1 g/day.

### Fecal sample collection

Subjects were given a fecal kit box as a place to store fecal samples a day before collection day. The fecal kit box containing sterile fecal bottle, trail paper, rubber gloves, masks, and ice gel was provided by the laboratory. Ice gel contained in the fecal kit box was previously frozen before used. The ice gel serves to keep the fecal sample cold ( $<10^{\circ}\text{C}$ ) and it was transferred to the laboratory within 1 h [13].

### SCFA and stool pH analysis

SCFA compounds were measured using the method from Salazar *et al.* (2014) [19]. Analysis of SCFA compounds using gas chromatography (GC) tools (Shidmadzu, GC series 2010 plus) with injector specifications of  $240^{\circ}\text{C}$ , RTX-Wax column temperature and column length  $145^{\circ}\text{C}$  and 30 m, diameter 0.25, column flow 0.80 min using a helium carrier gas and using a flame ionization detector (FID) at  $240^{\circ}\text{C}$ . A total of 0.2 g of fecal samples added with 1 ml distilled water, vortex, sonification for 20 min, and then centrifuged at 13,000 rpm for 5 min at  $4^{\circ}\text{C}$ . After that, supernatant

was transferred and centrifuged again at 13,000 rpm for 5 min at 4°C. Then, 1 ml of supernatant was collected and ready to be analyzed with GC equipped with an FID detector and capillary column. Stool pH was analyzed using a pH meter (pH Spear Eutech). After calibration using buffer, the probe was directly taking into the stool sample and kept until a stable measured value.

### Nutritional status

Nutritional status of the study participants was assessed by three anthropometric indices that are generally used as measurement for children's nutritional status: Length-for-height for age z-score (HAZ), weight-for-age z-score (WAZ), and weight-for-height z-score (WHZ) [17]. Weight and height were measured monthly. A cutoff of  $<-2SD$  was used to distinguish normal children from those stunted (HAZ  $<-2SD$ ) or underweight (WAZ  $<-2SD$ ) or wasted (WHZ  $\leq 2$ ). Weight measurements were taken to the nearest of 0.1 kg, using a standardized 20 kg infant digital scale and measurement of height was taken to nearest of 0.1 cm, using measuring board for children under 2 years and microtomies for children over 2 years.

### Statistical analysis

Statistical analyses were performed in R software (Version 2.15.3). Baseline characteristics of the study were compared by independent t-test analysis. Data are presented as mean  $\pm$  SD. Data were tested for normality using Shapiro–Wilk test. The comparisons between the groups were analyzed using t-test. Values with  $p < 0.05$  were considered statistically significant.

### Ethical Consideration

The study was conducted in accordance with declaration of Helsinki. Parents or legal guardians were fully informed about the aim of the study and signed informed consent was obtained from at least one parent or legal guardians. Protocol was approved by the Ethical Committee, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia (approval date: November 25, 2019; reference number: KE/FK/1388/EC/2019).

### Results

During this study period, 40 stunting children under 5 years old agreed to participate in this study, but during consumption period one of the subjects

was dropped out as the subject refused to participate. There were no complains and/or adverse event by the intervention during and after intervention. The level of compliance was in a good category (mean  $\geq 80\%$ ) because the mothers of the subjects felt that since consumption period, their children's appetites have become better, so that the mothers almost never forget to give these products to their children. As a result, a total of 39 subject completed this clinical study (symbiotic group = 19 and placebo = 20). Figure 1 shows the flow diagram showing the subject's progression during this study period.

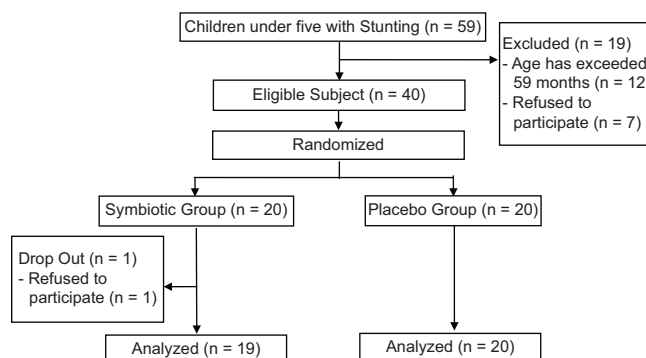


Figure 1. Recruitment of Study Subject: Flow chart

### Characteristic of subject

There was no significant difference on both groups after baseline characteristic analysis. Therefore, it can be concluded that the subjects had the same characteristics at the baseline. Characteristics of research subjects including age, sex, and nutritional status of the subjects are shown in Table 1.

Table 1: Baseline characteristic of subject

| Characteristics         | Symbiotic group (n = 19) | Placebo group (n = 20) | P     |
|-------------------------|--------------------------|------------------------|-------|
| Sex                     |                          |                        |       |
| Male                    | 12                       | 13                     |       |
| Female                  | 7                        | 7                      |       |
| Age, month              | 26 $\pm$ 8.34            | 29 $\pm$ 5.78          | 0.194 |
| Weight, kg              | 8.5 $\pm$ 0.94           | 9.0 $\pm$ 0.82         | 0.103 |
| Height, cm              | 78.96 $\pm$ 5.4          | 80.9 $\pm$ 4.55        | 0.241 |
| Weight for age (WAZ)    | -2.97 $\pm$ 0.6          | -3.1 $\pm$ 0.36        | 0.561 |
| Height for age (HAZ)    | -2.8 $\pm$ 0.54          | -3.0 $\pm$ 0.70        | 0.384 |
| Weight for height (WHZ) | -2.15 $\pm$ 0.6          | -2.0 $\pm$ 0.63        | 0.524 |

\*Independent t-test, significant if  $P < 0.05$ . Value is expressed as mean  $\pm$  SD.

### Short chain fatty acid and stool pH

The acetate, propionate, and butyrate levels found to be increase on fecal samples of both groups, but the most abundant was acetate. The results of SCFA profile are shown in Table 2. The acetate, propionate, and butyrate in symbiotic group after intervention were significantly increased. Conversely, in placebo group, significant different is only found in the concentration of acetate. The slightly significant difference between groups after consumption period on acetate, propionate, and butyrate levels can be seen when symbiotic and placebo group are compared.

**Table 2: The difference between and within groups on short-chain fatty acid**

| SCFA level          | mmol/g Feces (Mean $\pm$ SD) |                  | p-value <sup>a</sup> |
|---------------------|------------------------------|------------------|----------------------|
|                     | Symbiotic group              | Placebo group    |                      |
| Acetate             |                              |                  |                      |
| Before intervention | 10.02 $\pm$ 3.44             | 10.75 $\pm$ 2.03 | 0.427                |
| After intervention  | 17.10 $\pm$ 6.88             | 12.44 $\pm$ 3.61 | 0.000*               |
| Propionate          |                              |                  |                      |
| Before intervention | 4.8 $\pm$ 1.27               | 4.79 $\pm$ 0.71  | 0.976                |
| After intervention  | 7.7 $\pm$ 2.05               | 5.20 $\pm$ 1.66  | 0.000*               |
| Butyrate            |                              |                  |                      |
| Before intervention | 5.74 $\pm$ 1.50              | 5.97 $\pm$ 0.92  | 0.574                |
| After intervention  | 7.47 $\pm$ 1.76              | 6.12 $\pm$ 1.16  | 0.008*               |

Data are presented as mean  $\pm$  SD, \*Significant if  $P < 0.05$ , <sup>a</sup>Independent t-test

Figure 2 shows the significant difference in stool pH between groups after 90 days intervention with  $p = 0.000$ . The symbiotic group had lower stool pH after the intervention, which was  $6.31 \pm 0.16$ , while the placebo group was  $6.74 \pm 0.41$ . The decrease in pH in the colon is due to the presence of organic acids produced by acid-producing bacteria (acetate, propionate, and butyrate) which stimulate motility in the colon.

### Nutritional status

The comparison results from before and after consumption period in both groups are shown in Table 3. The average of z-score was on under nutrition category for WAZ and stunting category for HAZ ( $\leq -2$  SD), while for HAZ the average of z-score was into normal category ( $> -2$  SD). However, there was a significant increase for WAZ, HAZ, and WHZ on symbiotic group but not on placebo group.

**Table 3: Differences comparison on nutritional status of subjects before and after intervention in both group**

| Nutritional status                  | Symbiotic group  | Placebo group    | p-value <sup>a</sup> |
|-------------------------------------|------------------|------------------|----------------------|
|                                     | Mean $\pm$ SD    | Mean $\pm$ SD    |                      |
| Weight-for-age z-score (WAZ)        |                  |                  |                      |
| Before                              | -2.96 $\pm$ 0.62 | -3.06 $\pm$ 0.36 | 0.566                |
| After                               | -2.10 $\pm$ 0.65 | -2.80 $\pm$ 0.53 | 0.001                |
| Length/height-for-age z-score (HAZ) |                  |                  |                      |
| Before                              | -2.79 $\pm$ 0.55 | -2.97 $\pm$ 0.72 | 0.409                |
| After                               | -2.12 $\pm$ 0.48 | -2.83 $\pm$ 0.75 | 0.003                |
| Weight-for-height z-score (WHZ)     |                  |                  |                      |
| Before                              | -2.14 $\pm$ 0.70 | -2.00 $\pm$ 0.64 | 0.485                |
| After                               | -1.35 $\pm$ 0.75 | -1.73 $\pm$ 0.90 | 0.104                |

Data are presented as mean  $\pm$  SD, <sup>a</sup>Significant if  $P < 0.05$ , an independent t-test

In Table 4, a detailed result of nutritional changes between symbiotic and placebo groups can be seen. When compared, a significant difference between groups after intervention on WAZ and HAZ is found. However, there was no significant difference on WHZ score between compared groups (Table 4).

## Discussion

Stunting is a significant pediatric health problem worldwide that contributes to morbidity and mortality in children under 5 years of age [20]. A chronic syndrome causes inflammation of the small intestine, known as PEE in which an altered gut microbiota or dysbiosis and growth failure were found [21]. Dysbiosis refers to an altered gut microbiota composition that could lead to stunting even in the face of adequate food intake. The previous study shows that gut microbiota on stunting children is predominant in bacteria belonging to the inflammation taxa [6]. In this study, *L. plantarum* DAD-13 as a probiotic agent and FOS as a prebiotic agent were used. Previously, a study reported that 20 out of 20 healthy adolescent experienced an increase in the amount of *L. plantarum* after consuming the probiotic *L. plantarum* DAD-13 for 2 months. This shows that *L. plantarum* DAD-13 can live in the human gastrointestinal tract and has a beneficial impact on health. These results are in line with another research which states that *L. plantarum* DAD-13 is a strain that can survive in the digestive system, resistant to bile salt and gastric juice, and found in human feces who consume it [22]. FOS can increase *Bifidobacterium* that has an important role in human gut health. The decrease or loss of *Bifidobacterium* in the human gut indicates that they are not healthy [23]. *Lactobacillus* and *Bifidobacterium*, which can decrease inflammation, strengthen gut barrier function, inhibit pathogens, and mediate other beneficial effects under certain

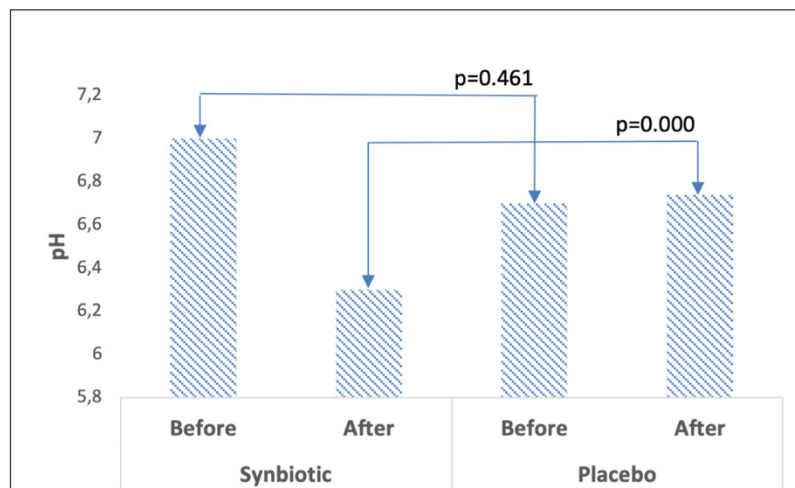


Figure 2: The different in stool pH between groups before and after intervention

**Table 4: Nutritional status (WAZ, HAZ, and WHZ)**

| Variable | Symbiotic group     |                   |                      |                     | Placebo Group       |                   |                      |                     |
|----------|---------------------|-------------------|----------------------|---------------------|---------------------|-------------------|----------------------|---------------------|
|          | Increment (subject) | Average (Min-max) | Decrements (subject) | Average** (Min-max) | Increment (subject) | Average (Min-max) | Decrements (subject) | Average** (Min-max) |
| WAZ      | 19 (100%)           | 0.87 (0.34–1.81)  | -                    | -                   | 13 (65%)            | 0.45 (0.02–1.54)  | 7 (35%)              | -0.08 (-0.13–0.01)  |
| HAZ      | 18 (94.7%)          | 0.71 (0.14–1.74)  | 1 (5.3%)             | -0.13 (-0.13–0.13)  | 16 (80%)            | 0.21 (0.01–1.01)  | 4 (20%)              | -0.11 (-0.2–0.07)   |
| WHZ      | 18 (94.7%)          | 0.84 (0–2.06)     | 1 (5.3%)             | -0.13 (-0.13–0.13)  | 10 (50%)            | 0.75 (0.05–2.17)  | 10 (50%)             | -0.21 (-0.56–0)     |

\*Data are presented as n (%); \*\*Unit value Standard deviation.

conditions, are deficient in stool from undernourished children.

Therefore, due to their favorable effect on the development of beneficial intestinal bacteria, the administration of prebiotics may participate in inhibiting the development of inflammation-inducing pathogens [24], [25]. Based on the previous study, it was revealed that overall the gut microbiota of stunted children was mostly consist of fluorogenic bacteria belonging to the Proteobacteria phylum, whereas those of children who were not stunted were mostly belong to probiotic species such as *Bifidobacterium longum* [2]. This shows that *L. plantarum* DAD-13 can reduce pathogens in the gut microbiota of specific group of subjects [26]. Fecal microbial communities from both undernourished cohorts, including increased proportions of pathogenic taxa within Proteobacteria, such as *Enterobacteriaceae*, *Escherichia*, *Klebsiella*, and *Shigella*, as by other studies [25], [2]. It should be noted that similar pattern of increased Proteobacteria proportions with decreased microbial diversity is found in inflammatory bowel disease patients. On the other hand, genera containing potentially beneficial organisms are depleted in the undernourished gut.

Symbiotic has both probiotic and prebiotic properties and was created to overcome possible difficulties in the survival of probiotics in the gastrointestinal tract [27]. Therefore, an appropriate combination of both components in a single product should ensure a superior effect, compared to the activity of the probiotic or prebiotic alone, especially to cure the inflammation. The main aim of symbiotic is to stimulate the growth and activity of beneficial bacteria in the gastrointestinal tract, which confers a desirable health benefit to the host. Through mechanisms of antagonism (the production of antimicrobial substances) and competition for epithelial adhesion and nutrients, the intestinal microbiota acts as a barrier for pathogens. The gut microbiota plays an important role in metabolizing undigested protein and carbohydrates which results in SCFA, namely: Acetate, propionate, and butyrate which are subsequently used by the host as a source of energy [28]. As a result of the fermentation of carbohydrates, *Bifidobacterium* or *Lactobacillus* may produce some compounds inhibiting the development of gastrointestinal pathogens, as well as reducing the intestinal pH [29]. The pH reflecting the environment acidity due to microbial metabolites [30]. There was an increase in stool pH on stunting children. Based on the previous study, elevated fecal pH indicates a mark change in the gut microbiota, specifically a profound reduction on probiotic species [31]. Similar

with the present study that reported after 90 days of consumption, *L. plantarum* DAD-13 and FOS resulted in the decrease a fecal pH.

The Bifidobacterium genus showed tolerance behavior toward the change of SCFA level and lower fecal pH. The low levels of propionate and butyrate were associated with inflammation [32]. Based on SCFA analysis, the level of acetate, propionate, and butyrate was significantly increase on symbiotic group after consuming symbiotic powder for 90 days. The synergy between probiotics and prebiotics can also reduce the pH of the intestinal lumen and stimulate cell proliferation to expand the surface of mineral absorption thereby increasing mineral bioavailability and leads to the increase of nutritional status [33]. The reported result is in line with this study where nutritional status (WAZ, HAZ, and WHZ) was significantly increased after consumption periods of 90 days. SCFAs are a subset of fatty acids produced by the gut microbiota during the fermentation of partially and non-digestible polysaccharides [34]. Acetate is a net fermentation product for most gut microbiota, whereas the production of butyrate and propionate are by specific bacterial species. A previous study investigated the effect of diet pattern, including the intake of fibers and iron, toward microbiota composition [35]. The study reported that diet pattern influences the microbiota composition, gut SCFA levels, gut motility, and strengthen the gut barrier functions. SCFAs are metabolite of microbial fermentation that derived from bacterial-dependent hydrolysis of fiber. SCFAs are estimated to contribute 6%–10% of total energy requirements and the contribution is expected to be higher for humans consuming high-fiber diets and herbivorous species. To acquire energy metabolism efficiency, the communication between the gut and peripheral organs (pancreas, liver, adipose tissue, and brain) is crucial. Information about nutritional status in the gut is relayed by various signals, including gut derived hormones such as glucagon-like peptide-1 (GLP-1). Transient postprandial increases in GLP-1 influence metabolism such as promoting insulin secretion (incretin effect), inhibiting the gastric emptying, and increasing satiety. The GLP-1 secretion from enteroendocrine L cells can be stimulated by consuming sugars, amino acids, and long-chain fatty acids. Dietary supplementation with fermentable fibers has been shown to increase GLP-1 levels in rodents and humans, where SCFAs can stimulate GLP-1 secretion *in vitro*. Thus, it has been suggested that by producing SCFA, gut microbiota can increase GLP-1 level. The absence of microbially produced SCFAs in germ-free colon results in

significantly higher plasma GLP-1 levels in mice. As an adaptive reason in promoting nutrient absorption, this colonic-derived GLP-1 seems to slow down the transit time in small intestine [36].

## Conclusions

The study demonstrated that 90 days of consuming synbiotic powder which consists of *L. plantarum* DAD-13 and FOS could contribute on the increase of SCFA (acetate, propionate, and butyrate) levels and lowering fecal pH. These alterations can decrease the inflammation process and stimulate cell proliferation to expand the surface of mineral absorption thereby increasing mineral bioavailability and lead to the increase of nutritional status (WAZ, HAZ, and WHZ) on stunted children.

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