Clinical assessment and cytokines level in constipation-predominant irritable bowel syndrome participants treated with Lactobacillus-containing cultured milk drink

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Abstract

Background: **Gut dysbiosis is linked with the pathophysiology of irritable bowel syndrome (IBS). Manipulation of intestinal microbiota using cultured milk drinks may stimulate the immune system, hence providing beneficial support in IBS treatment. This study aimed to investigate the effects of cultured milk drink on clinical symptoms, intestinal transit time (ITT), fecal pH and cytokines in constipation-predominant IBS (IBS-C) as compared to non-IBS participants.**

Methods: **Each recruited participant was given three bottles of 125 ml cultured milk drink containing 10^9 cfu Lactobacillus acidophilus LA-5 and Lactobacillus paracasei L. CASEI-01 consumed daily for 30 days. At pre- and post-30-day consumption, fecal pH, ITT, clinical symptoms, IL-6, IL-8 and TNF-α levels were assessed. Seventy-seven IBS-C and 88 non-IBS were enrolled.**

Results: **Post-consumption, 97.4% of IBS-C experienced improvements in constipation-related symptoms supported by the significant reduction of ITT and decreased fecal pH ($p<0.05$). All pro-inflammatory cytokines were significantly lower in post as compared to pre-consumption of cultured milk drinks in IBS-C ($p<0.05$). There was significant reduction in the IL-8 and TNF-α levels in post- as compared to pre-consumption for the non-IBS ($p < 0.05$).**

Conclusion: **Cultured milk drink taken daily improved clinical symptoms and reduced cytokines, hence should be considered as an adjunctive treatment in IBS-C individuals.** (Acta gastroenterol. belg., **2021,** 84**, 585-591**).

Keywords: Irritable bowel syndrome, cultured milk products,IL-6, IL-8, TNF-alpha.

Introduction

Irritable bowel syndrome is a common functional bowel condition seen globally (1,2). To date, IBS is diagnosed based on symptoms' criteria with no evidence of structural abnormalities (2). Rome III Diagnostic Criteria define IBS based on recurrent abdominal pain or discomfort for at least three days/month in the past three months associated with two or more of the following symptoms: improvement with defecation, change in fecal frequency, or change in the fecal form. These criteria should be fulfilled with symptom's onset at least six months prior to diagnosis (3). Four subtypes of IBS based on Bristol Stool Form are IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), mixed IBS (IBS-M) and unclassified IBS (IBS-U). When an individual passing out hard stools > 25% of the time and loose stool less than 25% of the time, it is defined as IBS-C (4). The prevalence of IBS was about 10 to 15% among Malaysians as compared to 10 to 22% worldwide (5-7). Despite being common, the pathophysiology of this disorder remains uncertain. Risk factors in IBS-C include young age, psychological stress, improper diet and lack of exercise (8). Previous published literatures indicated that visceral hypersensitivity, serotonin dysregulation, gut-brain interaction and gut dysbiosis were the possible pathophysiology of IBS (9-11).

Probiotic is normally classified as health supplement that does not require any approval or licensing (12). Cultured milk drink is a type of probiotic, and a form of food product produced by bacteria fermentation of milk containing live bacteria such as *Lactobacillus sp.* and *Bifidobacterium sp.*, which has been scientifically proven to tackle gut dysbiosis, hence improves digestive health (13,14). Encouragingly, consumption of cultured milk drink that contains *Lactobacillus casei* L. CASEI-01 and *Lactobacillus acidophilus* LA-5 showed a consistent trend towards an improvement of IBS symptoms, specifically abdominal pain, bloating and constipation (13). Additionally, *Lactobacillus rhamnosus* may be a treatment option by protecting against epithelial barrier dysfunction in IBS (15). However, studies are lacking in investigating the immune status of individuals pre- and post-cultured milk consumption. Using probiotic, alteration of the intestinal microflora with therapeutic intention stimulates the mucosal immune system. In a previous trial, there was a corresponding normalization in the ratio of serum IL-10/IL-12 suggesting that the probiotic may help reduce the pro-inflammatory state of immune system associated with IBS (14). The adaptive immune response comprises of T and B lymphocytes that, when activated, generate effector responses of cytokines and antibodies. It also enhances innate immune response by amplification of host defense through cytokines in controlling inflammation (16,17). In comparison to the

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innate immune system, the adaptive immune system is highly specific and confers long-lasting immunity.

Researchers previously showed pro-inflammatory cytokines such as interleukin (IL)-6, IL-8, tumor necrosis factor-alpha (TNF-α), and IL-1β were elevated in the systemic circulation of patients with IBS (18). IL-6 has both pro-inflammatory and anti-inflammatory entities (19). IL-8 is a chemokine which is associated with inflammation and is increased by oxidative stress. TNF-α is a polypeptide cytokine with variable actions in immune system. Therefore, this study was embarked to assess the benefit of cultured milk drinks on clinical symptoms and explore the circulating immune responses in healthy and IBS-C participants.

Materials and methods

Institutional review board statement

This clinical trial received ethical approval from Universiti Kebangsaan Malaysia Medical Centre (UKMMC) Research Ethics Committee (Reference number: FF2017-214) and retrospectively registered at WHO International Clinical Trial Registry Platform on 16th November 2020 with clinical trial ID NCT04647045. Written informed consents were obtained from all patients above 18 years old.

Study participants

This is a single-blinded, non-randomized clinical trial study conducted from May 2017 to June 2018. The researcher who recruited the subjects knew which the subjects was receiving and the final results were revealed after the clinical trial was over. Participants who fulfilled the Rome III IBS-C criteria were recruited from the Gastroenterology clinic at Universiti Kebangsaan Malaysia Medical Centre, Kuala Lumpur. The probability (power) of the study was set at 0.8 and Type I error probability was set at 0.05. Prior data by O'Mahony et al. (2005) indicated that the difference in the response of matched paired was normally distributed with standard deviation of 7.4. If the true difference in the mean response of matched pairs is 2.5, then the study needs 71 pairs of subjects to be able to prove the null hypothesis. The sample size was calculated using the Power and Sample Size (PS3) software (Version 3.1.2, Nashville, USA) (20). A total of 165 participants were recruited for this study. We included healthy participants above 18 years old and IBS-C participants fulfilling the Rome III criteria. We excluded participants with age less than 18 years old; constip ation due to other medical illnesses such as diabetes mellitus, inflammatory bowel disease, hypothyroidism, colorectal cancer, neurological diseases, major depression, lactose intolerance, pregnancy, breastfeeding women and must not be on chronic opioids or anti-depressants. Participants must not consume any antibiotics, probiotic, prebiotic, symbiotic and/or laxatives less than two weeks before recruitment.

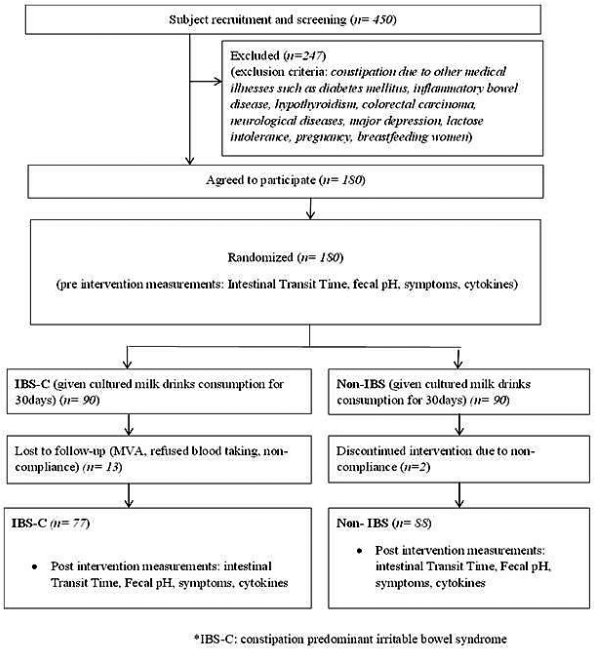

Figure 1. — The CONSORT flow diagram of study involving constipation predominant irritable bowel syndrome (IBS-C) versus non-IBS-C. * represents statistically significant at $p<0.05$.

Participants were required to consume three bottles of 125 ml cultured milk drink daily for 30 days (Fig. 1).

Each bottle contains 10^9 cfu *Lactobacillus acidophilus* LA-5 and *Lactobacillus paracasei* L. CASEI-01. In each 100 ml unit, it contains 4.06 g of fructose, 4.14 g of glucose, 0.29 g of sucrose, less than 0.1 g of maltose and 1.35 g of lactose. Cold chain was maintained below 10°C during storage, transportation and distribution. Prior to consumption of the cultured milk drinks, fecal and blood samples from the participants were obtained to determine their baseline fecal pH and serum cytokines level respectively. Each of the participants answered a food frequency questionnaire to assess their dietary profile (data are not presented in this paper) and the Garrigues Questionnaire to evaluate their clinical symptoms and physical activity. The same parameters were taken following 30 days of cultured milk drinks' consumption. The participants were reminded by phone via short message service (SMS) daily to ensure compliance and was asked to return all empty bottles of cultured milk drinks every week during the study period. Once a week, we called the participants to ensure that they did not develop any side effects of the probiotic consumption.

Protocol for intestinal transit time

To determine ITT, the participants took non-toxic food colorant of red carmine capsules (21). The time of capsule's consumption and the first appearance of red fecal sample were self-documented by the participants. Participants were required to send their fecal samples

after the ITT was determined and labeled as pre- (Day 0) and post-treatment (Day 30) with cultured milk drink.

Determination of fecal pH

Fresh fecal samples were sent in using standardized fecal containers and pH of fecal samples was measured using litmus pH paper at Day 0 and Day 30 of the study.

Constipation-related symptoms assessment

Garrigues Questionnaire was used to assess constipation-related symptoms (22). The components of the questionnaire included symptoms of straining during defecation, passing hard stool, number of bowel movements per week and incomplete emptying sensation after bowel movement. All questions were graded as never, sometimes, often or always. Pain was not included in the outcome because it is part of the Rome III diagnostic criteria.

Determination of serum cytokines

Five milliliter of peripheral venous blood sample were placed in a plain tube and centrifuged to prevent hemolysis. Serum was separated then stored in -80°C freezer until further analysis. Serum cytokines were measured by a commercially available enzyme-linked immunosorbent assay. Cytokine levels in serum samples were determined by a multiplex bead analysis (Milliplex HSTCMAG-28SK Human high Sensitivity T Cell Magnetic Bead Panel, Millipore, Watford, UK). Levels of the following cytokines were measured: TNF-α, IL-6 and IL-8. Each serum sample (50 µL) was incubated with antibody-coated capture beads overnight under agitation at 4°C. After washing, the beads were further incubated with biotin-labeled anti-human cytokine and chemokine antibodies for 60 minutes, followed by streptavidin-phycoerythrin incubation for 30 minutes. Finally, the beads were washed and analyzed in Luminex 200 (Luminex Corp, Austin, TX, USA). Standard curves of known concentration of recombinant human cytokines were used to convert a fluorescence unit to a cytokine concentration unit. Minimum detectable concentration was based against the reported value of the kit's manual. Data were stored and analyzed using Xponent 3.1 software (Luminex Corp, Austin, TX, USA).

Table 1. — **Demographic and socioeconomic details of constipation predominant irritable bowel syndrome (IBS-C)**

Parameter	IBS-C	Non-IBS
Total number of subjects (n)	77	88
Gender, n (%)		
Female	23 (29.9%)	50 (56.8%)
Male	54 (70.1%)	38 (43.2%)
Median age (IQR),years	27 (20-49)	28 (20- 50)
Ethnicity, n (%)		
Malay	50 (64.9%)	73 (83.0%)
Chinese	27 (35.1%)	15 (17.0%)
Smoker, n (%)		
Yes	1 (1.3%)	3 (3.4%)
No	76 (98.7%)	85 (96.6%)
Alcohol Intake, n (%)		
Yes	0 (0.0%)	0 (0.0%)
No	77 (100.0%)	88 (100.0%)
Fibre Intake, n (%)		
Low	40 (51.9%)	49 (55.7%)
Medium	33 (42.9%)	37 (42.0%)
High	4 (5.2%)	2 (2.3%)
Physical Exercise, n (%)		
Never	35 (45.5%)	34 (38.6%)
Sometimes	36 (46.8%)	44 (50.0%)
Habitually	6 (7.7%)	10 (11.4%)

IQR - Inter quartile range

Statistical analysis

All variables were tested for normality using the Statistical Package for the Social Sciences software version 22.0 (SPSS Inc. Chicago, IL). General linear models for repeated measures were used to analyze pre- and post-intervention between different groups. A p-value < 0.05 was considered as statistically significant.

Results

Demographic and socioeconomic details

A total of 450 participants screened, and 180 have agreed to take part. However, 15 participants defaulted intervention and were lost to follow up. Among the 165 participants, median age of the 77 IBS-C participants at 27 years old (IQR 20-49) was comparable with 88 non-IBS participants with a median age of 28 years old (IQR 20- 50) consort diagram is in Figure 1. The majority of IBS-C participants were male (70.1%), whereas non-IBS participants were female (56.8%). Among the participants involved, they were predominantly non-smokers and non-alcohol drinkers and the major ethnicity recruited was Malays. In regards to their physical activity and dietary intake, the majority claimed to have sometimes exercised and took low fiber diet. The results are shown in Table 1.

Intestinal Transit Time

The baseline ITT in the non-IBS group was within normal range (15.73 ± 9.28 hours), whereas in the IBS group, it was significantly longer (45.82 ± 28.89 hours) ($p < 0.05$). With the consumption of cultured milk drinks, both groups had significant reduction of ITT. It showed significant improvement in IBS-C and non-IBS from 45.82 ± 28.89 to 30.64 ± 20.07 hours and 15.73 ± 9.28 to 10.82 ± 5.34 hours respectively ($p < 0.05$) (Fig. 2).

Stool pH

Assessment of fecal pH initially was similar in both groups. Post 30-day consumption, fecal pH was

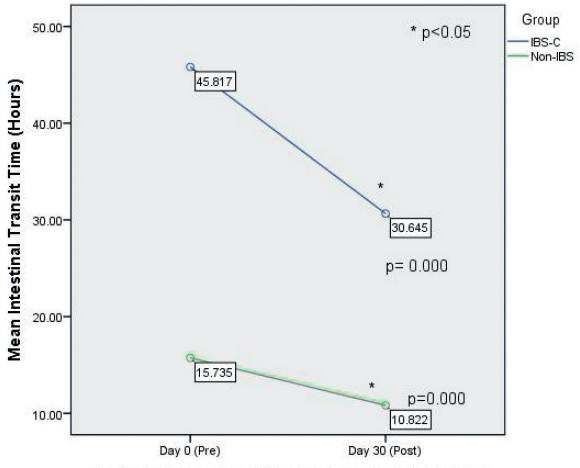

Figure 2. — Mean comparison of end-point measures (intestinal transit time) between IBS-C versus non-IBS (healthy) group. * represents statistically significant at p<0.05.

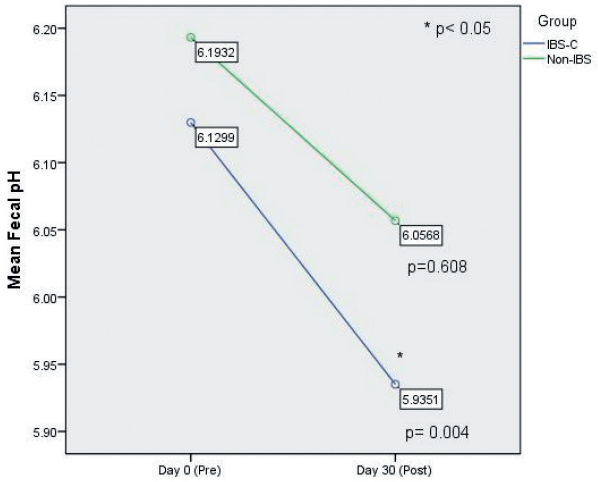

Figure 3. — Mean comparison of end-point measures (fecal pH) between IBS-C versus non-IBS (healthy) group. * represents statistically significant at p<0.05.

significantly reduced in IBS-C participants from 6.13 ± 0.57 to 5.94 ± 0.37 ($p < 0.05$).

There was no significant reduction in the fecal pH in non-IBS group in pre- and post-consumption of cultured milk drinks (Fig. 3).

Constipation-related symptoms assessment

Modified Garrigues constipation questionnaire included symptoms of straining during defecation, passing hard stool, number of bowel movements per week and incomplete emptying sensation after bowel movement. Following 30 days of cultured milk drink's consumption, 97.4% of the participants claimed that they had no constipation. In Figure 4 (a) shows there was reduction from 5% to 0% in those who responded as 'always' having to strain during defecation. While, there was an increase from 44% to 65%, who never have been straining. For

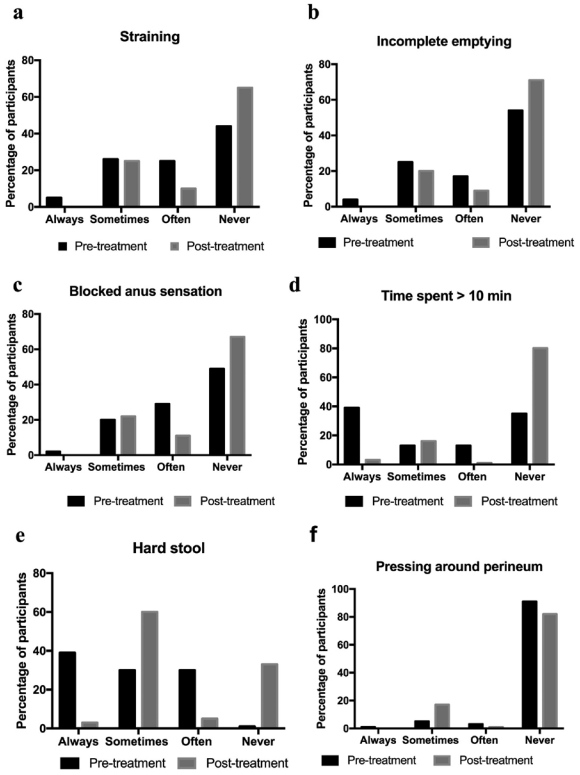

Figure 4. — Histogram showing the percentage of participants with constipation-related symptoms in constipation predominant irritable bowel syndrome (IBS-C) group (a) straining, b) incomplete emptying, c) blocked anus sensation, d) time spent >10 minutes, e) passing out hard stool, f) pressing around perineum).

incomplete evacuation, there was reduction from 17% to 9% for participants that responded as 'often' (more than 25% of time) post- as compared to pre-treatment. Comparable results were observed for other symptoms like anal blockage sensation and the need to spend more than 10 minutes to defecate. Post-intervention, there was a reduction from 29% to 11% of participants who 'often' experienced anal blockage. Whereas, a drop from 39% to 3% of the participants was still required to spend longer time to defecate following treatment. Figure 4(e) shows pre-treatment, the highest percentage (38.9%) of participants complained they 'always' having hard stool and the lowest was among those responded as 'never' had hard stool. Post-intervention, 2.6% responded as 'always' and 32.5% 'never' had hard stool. Lastly, 91% of the participants did not press around the anus to defecate during pre-treatment and following treatment; 82% never had to press around anus, 17% sometimes (less than 25% of time) and 1% often (more than 25% of time) as seen in Figure 4 (f).

Cytokines level

Compellingly, the IL-8 level was significantly reduced from 2.82 ± 3.34 pg/ml (pre-consumption) to 1.83 ± 1.82 pg/ml (post-consumption) in the non-IBS group

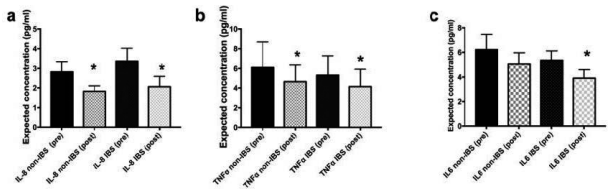

Figure 5. — Histograms showing the expected concentration of: a) interleukin 8 (IL-8), b) TNF- α and c) IL-6, between pre- and post-non-IBS (healthy) and pre- and post- IBS-C groups. * represents statistically significant at $p<0.05$.

($p < 0.05$) (Fig. 5 a). Significant reduction of IL-8 was also observed in IBS-C group from 3.35 ± 4.12 pg/ml to 2.06 ± 3.3 pg/ml ($p < 0.05$). Similar findings were seen in TNF-α levels, whereby it was significantly reduced in post- as compared to pre-consumption in both IBS-C and non-IBS groups ($p < 0.05$) (Fig. 5 b). The level of IL-6 was significantly lower in post as compared to pre-consumption of cultured milk drinks only in IBS-C group ($p < 0.05$) (Fig. 5 c). The results are shown in Figure 5.

Discussion

Rome III criteria constitutes useful tool in this study formally diagnose irritable bowel syndrome (1, 2). These criteria focus more on recent disease severity but the quantification of the IBS symptoms is still subjective. We utilized a single strain of probiotic *Lactobacillus spp.* to assess the symptom response and cytokines in IBS-C subjects. We have conducted this study at a tertiary center that covers an urban multi-racial population in the Klang Valley covering Cheras, Kuala Lumpur with the largest ethnic composition being Malay, followed by Chinese. Previous literature reported a mixture of findings in term of the duration of their findings. We chose 30 days or four weeks as the duration of the intervention and this was considered as a short intervention. Previous evidence had correlated cytokines and clinical symptoms by modulating gut microbiota using cultured milk drinks in IBS-C participants (18). The 30-day consumption period of cultured milk drinks was decided based on cost, compliancy and possibility of cytokines instability if given at a shorter or longer period. To the best of our knowledge, this study is the first in Malaysia to objectively assess the benefit of cultured milk drinks on clinical symptoms and explore circulating immune responses in both healthy and IBS-C participants, at baseline and at post-consumption of cultured milk drinks. A recent study provided guidelines on the use of probiotics in health care practice (23). Based on the Garrigues questionnaire, the majority (97.4%) of IBS-C participants experienced improvements in constipation-related symptoms, namely straining during defecation, passing hard stool, number of bowel movements per week and incomplete emptying sensation after bowel movement were seen at the post- as compared to pre- consumption of cultured milk drinks. This finding was supported by findings from previous literatures using cultured milk drinks (14,24). Cultured milk drink, is a form of food product produced by bacteria fermentation of milk that had been reported to aid digestive movements, increase defecation frequency, reduce abdominal pain or discomfort and reduce flatulence (25,26).

We discovered the ITT was delayed with higher fecal pH in IBS-C as compared to the normal population. This finding is in contrast with the recent reported data whereby intraluminal pH determined by wireless motility capsule, was significantly lower in IBS as compared to the healthy control (27) Upon completion of four weeks of cultured milk drinks consumption, there was a significant improvement in the ITT and reduction in the fecal pH in both groups. This finding was consistent with our work, which has been recently published (28). Under an anaerobic condition, good microorganisms may produce short chain fatty acids such as butyrate, acetate, propionate of varying quantities by fermenting on non-digestible food ingredient also known as prebiotics. ITT, on the other hand, often serves as a primary food ingredient also known as prebiotics. ITT, on the other hand, often serves as a primary related to intestinal function (29). Data published suggested that constipated adults and healthy populations showed clinical improvement from ingestion of *Bifidobacterium lactis*, DN-173 010 (25). Additionally, adults with constipation have increased in defecation frequency and improvement in stool consistency following ingestion of 65 mL/day for four weeks of *Lactobacillus casei* Shirota (26). Therefore, dysbiosis is strongly linked to pathophysiology of IBS even though the mechanism is not well understood. Finally, a lower pH enhances peristalsis in the intestine and subsequently, might decrease the colonic transit time (30). However, it is not known whether reduction in fecal pH and ITT in our study was purely due to cultured milk drink effects or variation in the participants' day-to-day diet.

We chose the most common proinflammatory cytokines involved in many chronic diseases i.e. IL-6, IL-8 and TNF-α. The baseline levels of serum cytokines in IBS-C participants were high that signified activation of inflammatory process. These findings were compatible with past studies that showed proinflammatory cytokines, especially IL-6, IL-8, TNF-α and IL-1β, were elevated in the systemic circulation of patients with IBS (16, 18). We found that the baseline level of IL-6 in our non-IBS (healthy) participants were higher than IBS-C participants. It seems reasonable to speculate that our healthy participants may not be as healthy as they claimed. There was no significant difference observed post-consumption of cultured milk drinks in IL-6 level among the healthy participants, possibly due to its complex regulation (31). A meta-analysis on IL-6 and IBS has suggested the use of IL-6 as a screening parameter to indicate low grade inflammation in IBS patients and the potential use of anti-inflammatory agent as part of personalized medicine (19). Elevated systemic cytokines in IBS patients produced by either T helper (Th) 1 or Th2 was not a

new breakthrough but data on the alteration of cytokine levels after intervention using cultured milk drink is a new compelling therapeutic alternative. Probiotics when used in the correct dosage and duration of treatment grants beneficial effect to the host at which in this case is constipation predominant type of IBS. Assembled information has shown that probiotics strengthened tight junction and stabilized intestinal permeability (32). This was achieved by alleviating immune dysregulation and improving cellular integrity to protect the colon in IBS. Current research did not look into the mechanism of how a specific strain of probiotic (e.g. Lactobacillus spp.) may modify the intestinal microbiota and acted as therapeutic role in IBS. These reassuring results, however, faced a few limitations where there was no placebo used as a control and therefore the clinical trial was not randomized. We also used cultured milk drinks that contain added sugar, which may have an effect towards blood-sugar level. Asymptomatic participants with borderline or impaired random blood sugar are encouraged to consume a cultured milk drink with low sugar contents. Another limitation for this study was that all subjects were not assessed for abdominal pain or discomfort pre- as well as post-treatment of probiotics.

Conclusions

Consumption of cultured milk drinks containing *Lactobacillus acidophilus,* LA-5 and *Lactobacillus paracasei,* L. CASEI-01 for 30 days in participants with IBS-C showed significant improvement in clinical assessments, ITT, fecal pH, IL-6, IL-8 and TNF-α levels. Consideration should be made to include cultured milk drinks containing 10^9 cfu *Lactobacillus acidophilus,* LA-5 and *Lactobacillus paracasei,* L. CASEI-01 for 30 days as an adjunct treatment in IBS-C.

Data availability

The raw data of this project will be released upon request.

Conflict of Interest

The authors declare that there is no competing interest.

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