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A PROBIOTIC FERMENTED MILK DRINK CONTAINING *LACTOBACILLUS CASEI* STRAIN SHIROTA IMPROVES STOOL CONSISTENCY OF SUBJECTS WITH HARD STOOLS

[1]Linde Tilley, [1]Katleen Keppens, [2]Akira Kushiro, [2]Toshihiko Takada, [2]Takafumi Sakai, [3]Mario Vaneechoutte and [4]Bart Degeest

[1]Groepspraktijk Heirnis (GP practice), Ghent, Belgium; [2]Yakult Central Institute for Microbiological Research, Tokyo, Japan; [3]Laboratory for Bacteriology Research (LBR), Department of Clinical Chemistry, Microbiology and Immunology, Universiteit Gent, Ghent, Belgium; and [4]Yakult Belgium, Brussels, Belgium

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ABSTRACT: ***The aim of this study was to investigate the effect of a fermented milk drink containing Lactobacillus casei Shirota (LcS) on the bowel habit (with emphasis on stool consistency) of subjects suffering from hard stools. Secondly, it was tested whether the probiotic strain (LcS), was able to survive throughout the gastrointestinal tract. A double-blind, placebo-controlled, randomized study was carried out over an eight-week period in subjects with symptoms of constipation (n=120). To all subjects, 65 ml/day of a probiotic fermented milk drink containing LcS or a placebo was administered. Patients completed a questionnaire to assess the consistency of their stools. In half of the study population, the survival of the probiotic strain (LcS) was tested using (i) culture and (ii) an LcS specific monoclonal antibody to identify the cultured colonies as LcS (ELISA). There was a significant decrease in stool hardening when consuming a fermented milk drink containing LcS. The observed clinical effect went hand in hand with the observed microbiological effect as the number of viable LcS bacteria in the faeces increased when consuming fermented milk drink containing LcS and decreased during wash-out.***

KEY WORDS: Constipation, Improved Stool Consistency, *Lactobacillus Casei* Shirota, Probiotic

Corresponding Author: Dr Bart Degeest, Yakult Belgium, Lenniksebaan 451, B-1070 Brussels, Belgium; Fax: +32 2 556 37 79; E-mail: bdegeest@yakult.be

INTRODUCTION

Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001). It is now understood that probiotic strains have specific properties and targets in the human intestinal tract. It is further realised that each probiotic strain, independent of its taxonomic position, is unique and thus the health benefits of each strain must be individually assessed (WGO, 2011). As implied by the definition, a basic property of all probiotics, is to remain viable until the site of action has been reached. Most often, this concerns survival during transit through the gut. The adverse effects of gastrointestinal tract conditions such as low pH, bile, and digestive enzymes, on the survival capacity of probiotic bacteria have been well documented (ILSI Europe, 2013). Various bacteria show different levels of tolerance to the gastrointestinal tract conditions, but all, if genuinely probiotic, display health-beneficial properties. An impressive number of reviews and books have documented the constantly evolving knowledge and state of the art in probiotics over the last decades. A few recent examples focus on health benefits in different populations (West et al., 2009; Chmielewska and Szajweska, 2010; Shanahan, 2010; Levin, 2011; WGO, 2011; Lupp et al., 2012; Hickson, 2013; Hungin et al., 2013; ILSI Europe, 2013; Sanders et al., 2013).

Bowel habit is a useful biomarker of gut health, especially of colonic function and is usually defined in terms of frequency of defecation, stool consistency and form and stool weight. One of the areas where probiotics might help to alleviate the symptoms is constipation and stool consistency. Constipation occurs due to many causes. One reason may be absence or decrease in the stimulation from the luminal side to the colonic mucosa. In animal experiments, lactic acid and short chain fatty acids such as propionate are shown to stimulate the colonic motility *in vitro* in the muscle strip preparation (Yokokura et al., 1977) and in-vivo intestinal motility test (Yajima, 1984; Mcmanus et al., 2002), respectively. Nowadays, much attention is paid to the symptoms of constipation or diarrhoea due to an increase in the incidence of irritable bowel syndrome of which 10 to 15 % of the population suffer from (Talley, 1999; Peppas et al.,

2008). Although the cause is complex, the disturbance of the intestinal microbiota may be associated with this disease (Si et al., 2004; Whelan and Quigley, 2013). Probiotics are expected to be a food adjunct that everybody takes easily and safely for the improvement of the constipation symptoms. A freeze-dried preparation of LcS, or a milk drink fermented with LcS has yet been used for patients with constipation. Both double blind placebo-controlled studies on Japanese (Matsumoto et al., 2006) and German (Koebnick et al., 2003; Krammer et al., 2011) subjects showed an increase in the number of bowel movements. Consistency is related to water content of the stool, which is normally 70-80% and constipated subjects generally pass harder stools with a water content of less than 70% (Cummings et al., 2004). It is the hardness and dryness of the stools that is thought to cause discomfort on defecation (Cummings et al., 2004; Walter et al., 2010). It has been shown that LcS influences stool consistency (Sakai et al., 2011).

The aim of this study was to support the earlier observations that the ingestion of LcS leads to the improvement of bowel habits, stool consistency in particular. Because it is assumed that in order to display the beneficial effect, the bacteria have to survive until they reach the site of action, a second part of this study focused on the detection of viable bacteria in the faeces of the volunteers.

MATERIALS AND METHODS

Study Population

A placebo-controlled, double-blind, randomized trial with two parallel arms was conducted with 120 subjects, aged 18 to 65 years old, suffering from mild constipation. Mild constipation was defined as four or less defecation moments per week and lumpy or hard stools in at least 25% of the defecations (*versus* constipation, defined as less than three defecation moments per week and lumpy or hard stools in at least 25% of the defecations, according to the Rome III criteria, Longstreth et al., 2006). The subjects were recruited in a general practitioners practice, in the area of Gent (Belgium). The exclusion criteria were: pregnancy, allergy to milk proteins, severe constipation (*e.g.* organic or neurological cause), regular use of probiotics within the preceding four weeks and regular use of laxatives, anti-diarrhoea medication, drugs with anti-cholinergic effect, or antibiotics (within a period of three weeks prior to the study). Subjects were specifically asked not to change their dietary habits during the study period.

This study was carried out according to the declaration of Helsinki. Subjects participating in this study were all volunteers. The written consent form was designed to assure the protection of subject's rights. The informed consent form was well documented, approved by the ethical committee and signed by all participants. Out of 120 subjects, 113 completed the clinical part of the study. At the first examination, the demographic data and medical history (including risk factors such as smoking and dietary factors) of the subjects were assessed by a questionnaire. The initial examination was followed by a run-in period of two weeks and an intervention period of four weeks. The final evaluation was carried out after two weeks follow-up period.

Study Formula

During the intervention period, the subjects were administered 65 ml/day of a probiotic fermented milk drink containing at least 6.5 X 10^9 cfu LcS, or 65 ml/day placebo. All ingredients were identical in the treatment and placebo products, except for the content of LcS. Placebo and the subjects or the investigators could not distinguish treatment product from each other. Other nutrient values were: 1.3 g of protein, 0.004 g of fat and 18.0 g of carbohydrates per 100 ml, and 580 mg of lactic acid.

Measurements

During the pre-ingestion, ingestion and follow-up period, stool consistency was assessed, on a daily basis, by a seven-point scale (7: watery, no solid pieces, 6: irregular watery pieces, 5: soft blobs, 4: soft sausage, 3: hard shaped sausage, 2: very hard shaped sausage, 1: hard lumps) according to the Bristol Stool Form Scale (Heaton et al., 1992). Consistency was calculated as an average of the stool consistency scores. Other questionnaires (constipation evaluation and digestive health evaluation) were filled out on a weekly basis throughout the study period, in order to detect possible effects and in order to check compliance. The general well-being was checked and an overall assessment was done by the general practitioners after four weeks and eight weeks.

The faecal samples of sixty volunteers (half of the study population) were analyzed after the pre-ingestion (two weeks), ingestion (six weeks) and follow-up (eight weeks) period. Freshly voided faecal samples were kept at low temperature (4°C) during storage and transport. After confirming the condition of samples during transport, 0.4g to 0.6g of faeces was weighted, followed by adding 2g of dry-heated sterilised glass beads and PBS dilution buffer (nine times the weight of the sample). The suspension was mixed thoroughly to homogenize. This faecal suspension was considered as a 10-time dilution of the faecal sample and used as the start solution for the serial dilutions. Hundred µl of the different dilutions was inoculated onto Lactitol LBS Vancomycin (LLV) agar plates (Nikken bio medical laboratory, Kyoto, Japan) and cultured at 37°C for 3 days.

Colonies grown on the plates were first divided into different types according to their size, color, gloss and shape, before being subject to Gram staining and microscopic analysis. Colonies with a microscopic morphology similar to LcS were further examined by ELISA using a specific monoclonal antibody against LcS. The method used was described previously by Yuki *et al.*, 1999).

Statistical Analysis

The power of the current study was calculated based on the incidence levels of an earlier conducted constipation study (Koebnick et al., 2003). A total number of 104 subjects were

needed for the current study (dropout not taken into account). Statistical analysis of all experimental results was carried out using SAS. Depending on the validity of the data (number of subjects for a certain question), the Students t test, the Wilcoxon rank test or both were used. Subjects for whom the dataset was not complete were not included in the statistical analysis.

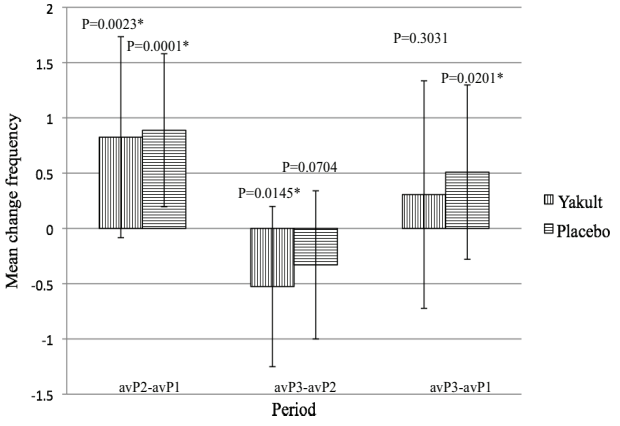
RESULTS AND DISCUSSION

In total, 113 subjects were enrolled to participate in the study. From this total population, 106 subjects (50 Yakult group, 56 placebo group) finished the clinical part of the study and 49 subjects (19 Yakult group, 30 placebo group) participated in the microbiological (faecal analysis) part of the study. In the pre-ingestion period, LcS was detected in 5 subjects (4 in the Yakult group and 1 in the placebo group), and faecal samples of 7 subjects (3 in the Yakult group and 4 in the placebo group) were not delivered in all three periods. Therefore, microbiological analysis was considered for 37 subjects (12 in the Yakult group and 25 in the placebo group). There were no differences between the Yakult and the placebo group with respect to age, body mass index, sex, general health status or dietary habits. Other questionnaires (as described in the materials and methods section), and the general well-being assessment did not show significant differences, but indicated a good compliance of the volunteers (data not shown).

For stool frequency, no significant difference was observed between the Yakult group and the placebo group, when comparing week by week or three periods (pre-ingestion, ingestion, follow-up).

When comparing mean differences during the above described three periods (within groups), a significant change with regard to stool frequency was observed between period 1 (pre-ingestion) and period 2 (ingestion), for both the Yakult and placebo group (Figure 1). Period 3 (follow-up) compared to period 2 (ingestion) showed a significant improvement for the Yakult group, but only a tendency of improvement for the placebo group (not significant).

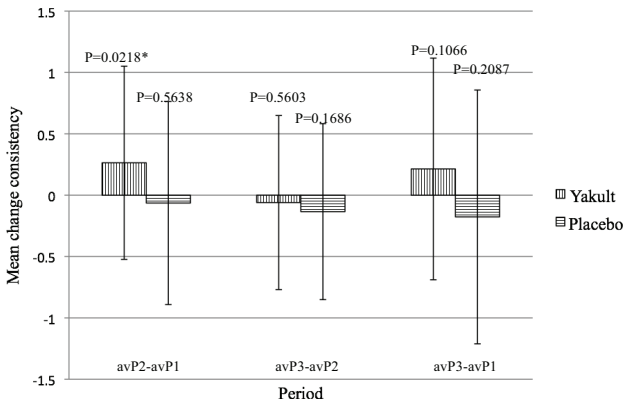
FIGURE 1. Changes of stool frequency within groups (expressed as number of defecations per day). Mean (av) differences were compared for three periods: P1, w1-w2 (pre-ingestion); P2, w3-w6 (ingestion); P3, w7-w8 (wash-out). Significant difference is marked with *.



For period 3 (follow-up) compared to period 1 (pre-ingestion), a significant difference was observed for the placebo group. Taken together, it is not possible to draw conclusions based on the observations for stool frequency.

The stool consistency at the beginning of the intervention did not differ between the Yakult and placebo group: the average Bristol stool consistency scale during weeks 1 and 2 was 2.832±1.198 in the Yakult group, and was 3.001±1.251 in the placebo group. Stool consistency has been calculated as a weekly average of the Bristol stool form scales. A week to week comparison showed no significant change in stool consistency (Bristol stool form scales). As shown in Figure 2, when the three periods (as described in the previous paragraph on frequency) were compared, a significant improvement in stool consistency was observed between period 2 and the first two weeks (period

FIGURE 2. Changes of stool consistency in Bristol stool form scales within groups. Mean (av) differences were compared for three periods: P1, w1-w2 (pre-ingestion); P2, w3-w6 (ingestion); P3, w7-w8 (wash-out). Significant difference is marked with *.

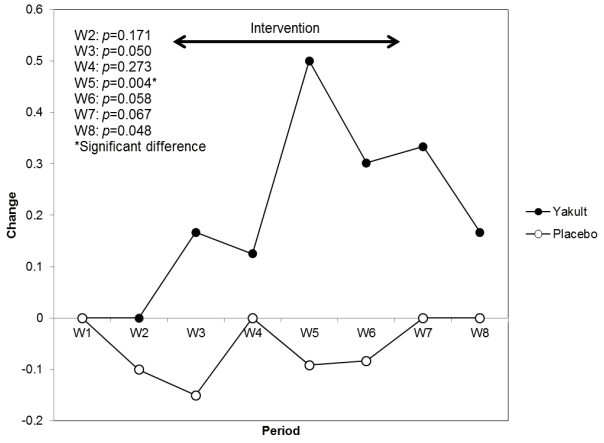


1) for subjects ingesting LcS. Stool became softer, more liquid after the ingestion of Yakult. The effect disappeared during the follow-up period. For subjects ingesting placebo, no changes were observed. Furthermore, a t-test comparison between the Yakult and placebo group demonstrated a significant effect when mean differences for the (i) ingestion period minus pre-ingestion period (avP2-avP1) (p=0.040) and (ii) wash-out period minus pre-ingestion period (avP3-avP1) (p=0.045 were compared, confirming that the ingestion of Yakult resulted in significant improvement of stool consistency in comparison to placebo.

In addition, a weekly comparison was made for the changes (i.e. difference w2-w1, difference w3-w2, …) from week 1 between both groups, using the Wilcoxon rank sum test adjusted by Bonferroni procedures. The median values plotted in the graph demonstrate a significant difference in week 5 (Figure 3). From week 3 to week 8, there is a clear tendency for LcS to be more effective to soften stool in comparison to placebo. Taken together, a significant effect of Yakult on stool consistency (improvement of Bristol stool form scales) was observed in this study. The effect was demonstrated within

the group of subjects taking LcS by comparison of stool consistency before and after ingestion and between the groups of subjects taking LcS and subjects taking placebo. This effect disappears during the wash-out period.

FIGURE 3. Evolution of changes of stool consistency, expressed in Bristol stool form scales (median values plotted in the graph).



Similar findings were observed in a study carried out with an identical fermented milk drink containing LcS (Sakai et al., 2011). Indeed, the daily consumption of a fermented milk drink containing LcS reduced the incidence of hard and lumpy stools in a healthy population. The Bristol stool form scale was also significantly improved. Two earlier studies with LcS already demonstrated a positive effect on bowel habits (Koebnick et al., 2003; Matsumoto et al., 2006). The onset of the effect became apparent from the second week of the intervention onwards. Next to the softer stools, also the defecation frequency increased in the studies mentioned. It has been observed before that the stool consistency, influenced by the intake of LcS, is important in the evaluation of the intestinal functioning of subjects suffering from mild constipation (Koebnick et al. 2003; Matsumoto et al., 2006). The methodology to study effects of probiotics as functional food products on the intestinal functioning, is still being debated (Cummings et al., 2004). Thereby, the methods to define a target group and adequate endpoints are the most decisive elements of a study, as these can directly influence the interpretation of the study outcome related to the possible benefits of such a product on the health status of a person. In the present study in Belgium and previous studies in Japan (Matsumoto et al., 2006) and Germany (Koebnick et al., 2003), the stool frequency has been used as the inclusion criterion and as one of the primary endpoints, together with the stool consistency, to evaluate the benefit of the ingestion of probiotics. However, it is worth notifying that while the previous studies showed the improvement of stool frequency, but not in the present study (no significant difference between test and placebo arm), all of three studies demonstrate the improvement of stool consistency regardless different populations. Based on this evidence, it is tempting to speculate that the stool consistency could be a better primary indicator than stool frequency not only to evaluate the effect of probiotics on intestinal functioning but also to select the target group. More recent studies confirm this thesis and show an improvement in stool consistency after the consumption of probiotic products (De Paula et al., 2008; Yang et al., 2008; Guyonnet et al., 2009; Sakai et al., 2011). The stool consistency, as part of the bowel habit, is considered as a principal biomarker (Cummings et al., 2004) to evaluate the gut health, in particular the colonic function. In addition to that, Lewis and Heaton (2004) have shown that whole gut transit time is more related to stool form than defecation frequency. Although an underlying mechanism of this benefit of LcS on stool consistency is unknown, a potential interaction between LcS or its metabolites and a system controlling faecal water content that is an important function of the large intestine, forms a promising hypothesis. People with constipation show a decreased motility of the colon. It has been shown that LcS counteracts constipation related complaints by causing changes in the intestinal microbiota. It has been demonstrated that not only the number of LcS bacteria increases in the faeces after consumption of LcS, but also the total number of lactobacilli and bifidobacteria (Matsumoto et al., 2006; Spanhaak et al., 1998). The number of bifidobacteria is hypothesized to be a marker of a healthy colonic microbiota (Mitsuoka, 1990). Furthermore, bifidobacteria seem to play an important role in

FIGURE 4. Recovery of LcS in the faeces after consumption of the fermented milk drink containing LcS (Yakult group).

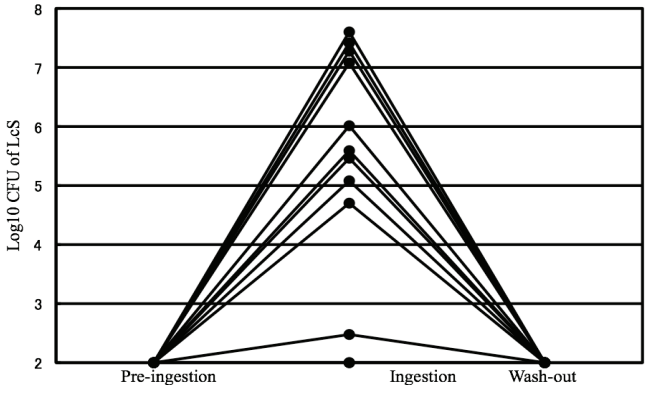
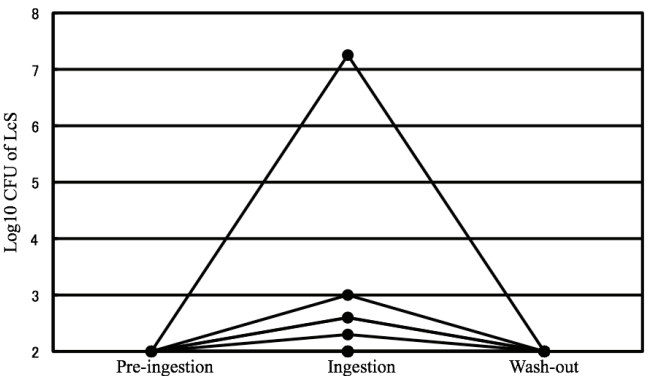


FIGURE 5. Recovery of LcS in the faeces after consumption of the placebo.



the regulation of water content of the gut contents (Picard et al., 2005). In addition to this effect, it has been demonstrated that lactobacilli and bifidobacteria produce metabolites, such as lactate and short chain fatty acids. These metabolites may increase the water content in the gut due to their osmotic effect.

Interaction between LcS and the enteric nervous system (ENS) in the gut also might be a possible mechanism for the detected effect of LcS on the stool consistency. The ENS is the collection of all neurons in the GI tract. It is estimated that there are 150 million nerve cells that comprise this so-called 'Gut Brain'. The ENS regulates through different mechanisms: motility, secretion, blood supply and immune reactions (Raj and Hirano, 1996).

Finally, it is hypothesized that ammonia and conjugated bile acids are involved in slowing down transit, resulting in constipation. Consuming a fermented milk drink containing LcS, however, clearly reduces microbial enzyme activity leading to xenobiotic substances (Spanhaak et al., 1998). Also the formation of other toxic compounds, such as ammonia and phenolic compounds is reduced (De Preter et al., 2004; Gibson et al., 2005; De Preter et al., 2007; De Preter et al., 2008). Most of the abovementioned possible explanations for the effect, in case of constipation, are based on the influence of LcS on the host microbiota. Consequently the bacteria have to reach the site of action alive. The latter was demonstrated in the second part of this study.

In order to confirm the survival of LcS in the gut, faecal samples of half of the study population were investigated. In the current report, the number of viable LcS bacteria is expressed as the logarithmic value of colony forming units (CFU) per g faeces. The logarithmic value of the detection limit for LcS is set as 2. When comparing Figure 4 and Figure 5, it is clear that in the Yakult group, the number of LcS bacteria increased after 4 weeks consumption and decreased again in the follow-up period. As expected, no increase was observed in the placebo group.

LcS was detected in the faecal sample of 11 subjects out of 12, and the logarithmic value of average CFU per g faeces was 5.2. As for the placebo group, LcS was not observed in 17 subjects out of 25. Unexpectedly, in the placebo group, LcS was found in a faecal sample of the ingestion period at low level in 7 subjects (Figure 4). One could speculate that contamination with LcS might have occurred during the dilution or cultivation process. LcS was detected at high CFU level (more than 7 $\log_{10}$ unit) in one volunteer. One could speculate that this volunteer consumed the fermented milk drink containing LcS during the ingestion period. No further investigation was done in the frame of this study.

In recent scientific literature, the methods to determine survival of probiotic bacteria are often criticized, due to the fact that samples can only be taken at the end of gastrointestinal tract (faeces). For LcS, however, next to *in vitro* data, many data have been accumulated to demonstrate the survival in the gastrointestinal tract of humans (Yuki et al., 1999; Lewis et al., 1997; Tuohy et al., 2007). This study confirms previously obtained results. Moreover, the viability of the bacteria in the gastrointestinal tract is linked to the clinical effect described in the first part of this study.

CONCLUSIONS

The ingestion of a fermented milk drink containing LcS leads to a significant effect on stool consistency. When consuming Yakult, stools of the subjects suffering from mild constipation, become less hard. The improvement is probably due to changes in the intestinal microbiota. It was confirmed again that LcS survives the gastrointestinal tract and reaches the intestines alive. The exact mechanism for the alleviation of the constipation symptoms is not yet fully elucidated, but needs to be investigated further.

CONFLICT OF INTEREST STATEMENT

This study was completely funded by Yakult. The authors Akira Kushiro, Toshihiko Takada and Takafumi Sakai, involved in the part on confirmation of the survival of LcS (Figures 4 and 5), are employees of the Yakult Central Institute for Microbiological Research, Tokyo, Japan. The involvement of these scientists was necessary due to the in-house knowledge and expertise for LcS detection. The author Bart Degeest is an employee of Yakult Belgium NV and was involved in the general coordination of the study and proofreading the manuscript.

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