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The effect of a probiotic blend on gastrointestinal symptoms in constipated patients: a double blind, randomised, placebo controlled 2-week trial

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RESEARCH ARTICLE

Abstract

Selected strains of lactobacilli and bifidobacteria are known to ameliorate constipation-related symptoms and have previously shown efficacy on digestive health. In this clinical trial, the safety and effectiveness of a probiotic blend containing lactobacilli and bifidobacteria were evaluated in adults with self-reported bloating and functional constipation. Constipation was diagnosed by the Rome III criteria. A total of 156 adults were randomised into this double-blind and placebo-controlled trial. Participants consumed the combination of *Lactobacillus acidophilus* NCFM (10^{10} cfu), *Lactobacillus paracasei* Lpc-37 (2.5×10^9 cfu), *Bifidobacterium animalis* subsp. *lactis* strains Bl-04 (2.5×10^9 cfu), Bi-07 (2.5×10^9 cfu) and HN019 (10^{10} cfu) (n=78), or placebo (microcrystalline cellulose) (n=78) for two weeks. After treatment the following were measured: primary outcome of bloating and secondary outcomes of colonic transit time, bowel movement frequency, stool consistency, other gastrointestinal symptoms (flatulence, abdominal pain, and burbling), constipation-related questionnaires (PAC-SYM and PAC-QoL) and product satisfaction. Faecal recovery of consumed strains was determined. The enrolled population was defined as constipated, however, the initial bloating severity was lower than in previous similar studies. No clinically significant observations related to the safety of the product were reported. Product efficacy was not shown in the primary analysis for bloating nor for the secondary efficacy analyses. The placebo functioned similarly as the probiotic product. In post-hoc analysis, a statistically significant decrease in flatulence in favour of the probiotic group was observed; day 7 (intention-to-treat (ITT): P=0.0313; per-protocol (PP): 0.0253) and on day 14 (ITT: P=0.0116; PP: P=0.0102) as measured by area under the curve (AUC) analysis. The mean AUC of all symptoms decreased in favour of the probiotic group, indicating less digestive discomfort. The study was registered at the ISRCTN registry (ISRCTN41607808).

Keywords: bifidobacteria, CTT, flatulence, lactobacilli, transit

1. Introduction

Constipation is a commonly diagnosed gastrointestinal disorder with an estimated prevalence of 12-19% in the general population (Higgins and Johanson, 2004; Locke *et al.*, 2000). Females and the elderly are at increased risk for constipation (Harris, 2005). Constipation results in lower quality of life and significant healthcare costs to the individual (Belsey *et al.*, 2010). The diagnosis of functional constipation and constipation-predominant irritable bowel syndrome (IBS-C) are based on similar symptoms, with IBS-C also being associated with abdominal pain or discomfort (Longstreth *et al.*, 2006). The cause of constipation is often unknown and likely multifactorial with physiological changes, psychological factors, and lifestyle influences identified as possible sources. The initial management of constipation-related symptoms is focused on evaluating lifestyle and diet variables as possible causes. If lifestyle modifications are unsuccessful in alleviating constipation, various medications may be prescribed. However, overall these medications have limited efficacy, are expensive, and may result in side effects, especially over long-term use. Thus, there is a need for alternative constipation treatments that are safe, effective, and cost-effective.

Bloating, as a subjective feeling of increased pressure within the abdomen, is a bothersome symptom possibly related to slowed transit, increased faecal mass in the colon and/or gas-production by the gut microbiota (Iovino *et al.*, 2014). A third of the general population and almost all IBS patients suffer from bloating (Longstreth *et al.*, 2006). In a large survey conducted in the USA on bowel symptoms among individuals with IBS-C and those with functional constipation, the subjective feeling of bloating was encountered 2.8 and 1.4 times a week (respectively) and approximately every second individual considered bloating to be a very or extremely bothersome symptom (Heidelbaugh *et al.*, 2015). Gastrointestinal illnesses may be associated with variance of or abnormalities in whole-gut or colonic transit time (CTT); for example, constipation is associated with an abnormally slow CTT, which in healthy individuals has been reported as having an average 30-40 hours but can range up to 70 hours (Bautista Casasnovas *et al.*, 1991; Kim and Rhee, 2012; Metcalf *et al.*, 1987). The severity of bloating is known to correlate positively with prolonged CTT (Raahave *et al.*, 2009). Thus, bloating may perform as a slow-transit-related symptom in constipation that can be affected by microbiota-targeting interventions and measured as a subjective feeling essential to the quality of life of the individual.

In recent years, probiotics (Fao/Who, 2001; Hill *et al.*, 2014) have been commonly used to alleviate symptoms in a variety of gastrointestinal disorders. It is hypothesised that probiotics help to maintain gut lumen homeostasis by suspending the growth of luminal pathogens and restoring the normal flora of the gut. As such, the use of probiotics for the relief of constipation-related symptoms, including bloating, is very promising. Numerous studies and meta-analyses have been published addressing the utility of probiotics for gastrointestinal health including constipation (Miller *et al.*, 2017c; Miller *et al.*, 2016). However, since probiotic efficacy is largely strain-specific, each specific strain must be individually tested in clinical trials.

The intestine has a complex population of beneficial bacteria which are essential for the host health including nutrient and vitamin availability and functional immunity (Turroni *et al.*, 2014). Bacteria from the genera *Bifidobacterium* and *Lactobacillus* are commonly found in the intestinal gut of humans and increasingly used in food and pharmaceutical applications to balance disturbed intestinal microflora and related dysfunction of the gastrointestinal tract. Selected strains of lactobacilli and bifidobacteria have traditionally been considered probiotics (Fao/Who, 2001; Hill *et al.*, 2014) and several lactobacilli and bifidobacteria strains have previously shown efficacy in digestive health. *Lactobacillus acidophilus* NCFM (NCFM) has been reported to modulate mu-opioid receptor expression and activity – linked to reduced visceral pain – in clinical and preclinical trials (Ringel-Kulka *et al.*, 2014; Rousseaux *et al.*, 2007). *Lactobacillus paracasei* Lpc-37 (Lpc-37) has been shown to reduce diarrhoea incidence in children (Hemalatha *et al.*, 2014). *Bifidobacterium animalis* subsp. *lactis* Bi-07 (Bi-07) has shown reduced diarrhoea incidence in toddlers, in addition to alleviation of constipation in adults (Bettler *et al.*, 2006; Favretto *et al.*, 2013). Studies with *B. animalis* subsp. *lactis* HN019 (HN019) have shown reduced diarrhoea incidence in children, reduced CTT and bowel symptoms (Dekker *et al.*, 2009; Hemalatha *et al.*, 2014; Waller *et al.*, 2011). When administered together with a prebiotic fibre, polydextrose, NCFM and Bi-07 have been able to reduce CTT (Magro *et al.*, 2014). While the combination of NCFM, Lpc-37, Bi-07 and *B. animalis* subsp. *lactis* Bl-04 (Bl-04) has been reported to reduce the incidence and duration of antibiotic associated diarrhoea and side effects of antibiotic use (Ouwehand *et al.*, 2014).

Given the promising clinical results of probiotics on gastrointestinal health to date, the objective of the current clinical trial was to evaluate the safety and effectiveness of a two-week supplementation of a probiotic blend containing selected strains of lactobacilli and bifidobacteria on bloating, CTT and other gastrointestinal symptoms in adults with self-reported bloating and constipation.

2. Materials and methods

Study design

This was a two-centre, randomised, double-blind, placebo-controlled, parallel-group, phase II study with two treatment arms. The study was conducted to determine the safety and efficacy of a two-week supplementation of a probiotic blend on abdominal bloating severity and other gastrointestinal parameters in an otherwise healthy adult population suffering from bloating and constipation. The study was conducted per globally accepted standards of good clinical practice (ICH, 1996), in agreement with the Declaration of Helsinki (WMA, 2013), and in accordance with local regulations for clinical research (additional Ethics and good clinical practice description in Supplementary Materials and methods). The primary outcome was subjective assessment of bloating incidence and severity. Secondary outcomes were CTT, defecation frequency, stool consistency, subjective assessment of constipation-related digestive symptoms in addition to bloating (flatulence, abdominal pain, and burbling), constipation-related questionnaires, and overall product satisfaction. Faecal microbial analysis was conducted for determining the product recovery. The study design is presented in Figure 1.

The eligible volunteers were adult participants aged between 18 to 70 years with functional constipation (FC) or IBS-C as per Rome III criteria (Drossman, 2006; Longstreth *et al.*, 2006), bowel movement frequency 1-3/week, stool consistency 1-2 according to the Bristol Stool Scale (BSS)

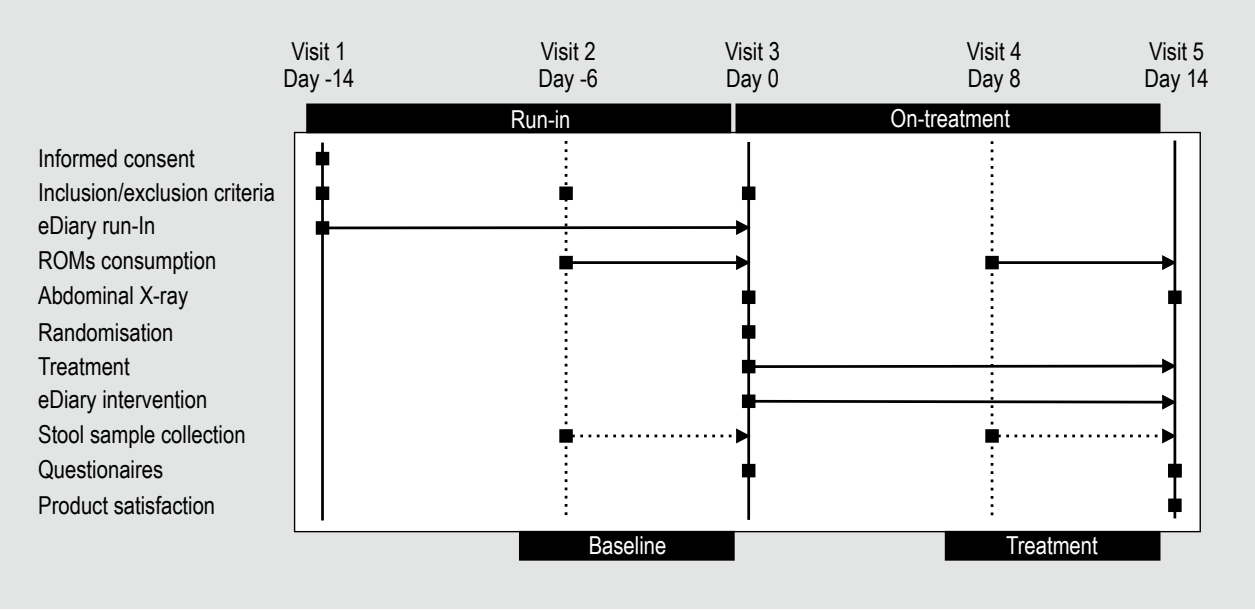

Figure 1. The study design and set-up. The following terms for different study periods were defined in the statistical analyses plan: run-in (day -14 to day -1), on-treatment (day 0 to day 13), baseline (day -7 to day -1) and treatment (day 7 to day 13). ROM = radio-opaque marker.

Form (Lewis and Heaton, 1997) for most spontaneously passed stools, and self-reported bloating at least twice a week. Although FC and IBS-C are different populations, we decided to combine these as this would cover self-reported constipation, which was studied earlier (Waller *et al.*, 2011). Detailed exclusion criteria are provided in supplemental material.

In total, 264 participants were pre-screened and 263 were enrolled after being fully informed and giving signed consent. The study consisted of a screening visit, a two-week run-in period, and a two-week on-treatment period. Participants who passed the initial screening and met the inclusion and exclusion criteria entered the run-in period. At screening, participants were given an android tablet computer with the electronic diary (eDiary) app (Mobanode, Limerick, Ireland), which included symptom survey, product compliance, and bowel movement frequency and stool consistency questions. During the run-in period, participants recorded relevant study data every day in the eDiary. After the first week of run-in (day -14 to day -7) the eligibility of the participants was re-assessed and those who met the inclusion criteria, continued in the study and were given radio opaque markers (ROMs) to be consumed daily for 6 days (day -6 to day -1). Participants who successfully completed the run-in period (including 100% compliance with ROM consumption, and 100% compliance with daily diary completion for stool frequency and consistency, and digestive symptoms) were randomised to receive the supplementation with either the active product or placebo (for composition see Supplementary Materials and methods).

Study procedure

Participants were randomised on a 1:1 basis to receive either the active probiotic blend or matching placebo. Randomisation was carried out by computer-generated block-randomisation lists. Participants were randomised to one of the two treatment groups in equal proportions. The block sizes varied randomly (4, 6, and 8). The randomisation was also seeded, with the seed only available to the statistician generating the randomisation list. The randomisation was provided by the statistician in a password-protected file. Danisco USA (Madison, WI, USA) produced, packaged, and labelled the investigational product (IP) according to the study code and treatment assignment following a strict double-blind procedure. All product dispensing occurred at the Atlantia Food Clinical Trials Ltd. study site in Blackpool, Cork, Ireland. All participants, site support staff, and investigators remained blinded to the product identity and group allocation. The study product was assigned to participants in chronological order. Detailed IP information provided in supplemental methods.

Participants were instructed to take one dose of study product per day, with breakfast, and to store the IP in a refrigerator (2-8 °C). Participants recorded their product consumption throughout the study in the eDiary. The time of administration of dosing was not recorded. At Visit 5 (day 14 of dosing), each participant was to return the completed eDiary and all unconsumed products, if any. Product compliance was assessed by calculating the number of returned capsules.

In addition, gastrointestinal symptom severity, defecation frequency and stool consistency were recorded daily in the eDiary, the international short self-administered physical activity questionnaire (Hagstromer *et al.*, 2006), covering the last 7 days, was assessed at the clinic at day 0 and 14, and EPIC-Norfolk food frequency questionnaire (FFQ) (Bingham *et al.*, 2001) was assessed once during the trial (day 0) to check for eligibility. The overall satisfaction with the study product's ability to relieve the constipation symptoms was assessed at day 14. A visit window of ±1 day was provided to the participants to add flexibility.

Safety assessment

Safety of the participants during the intervention was evaluated by adverse event (AE) enquiry during the weekly visits to the clinic (day 0, 8 and 14). The nature and frequency of AEs and serious adverse events (SAEs) were followed throughout the trial. The primary safety analysis was based on the frequency and severity of AEs. Absolute and relative frequencies of the number of AEs were reported for the categories: any, serious, by intensity, by relationship to study product, by action taken, and by outcome. SAEs were to be analysed similarly. The secondary safety analysis was based on vital signs and the clinical significance of the following variables: body temperature (ear, °C); systolic blood pressure (mmHg); diastolic blood pressure (mmHg); heart/pulse rate (beats/minute); height (cm); weight (kg); and body mass index (kg/m^2). The clinically significant findings of the vital signs were reported as frequency distributions and percentages.

Subjective assessment of constipation-related digestive symptoms

The primary efficacy variable was the change in average visual analogue scale (VAS) score for abdominal bloating in the second week of the intervention period (day 7 to 13). The change in average VAS scores from the Baseline period (day -7 to 1) to the so-called treatment period (day 7 to 13), controlling for baseline, were compared between the active and placebo groups. Participant assessment of bloating was measured each day during the run-in (day -14 to -1) and on-treatment (day 0 to 14) periods using a VAS scored from 0 to 100 in a similar manner to that used for individual symptoms scores in the IBS-symptom severity score (Francis *et al.*, 1997) and results were recorded in the eDiary. The average VAS was calculated as a sum of VAS scores (mm) divided by the number of days with VAS score data. The secondary outcomes for other constipation-related digestive symptoms in addition to bloating were flatulence, abdominal pain, and burbling. These digestive symptoms were reported as average VAS measured over the second week of intervention period (day 7 to 13), similarly to the primary outcome for bloating.

Colonic transit time

CTT was assessed using abdominal X-rays on day 0 and 14. Each participant ingested 20 ROMs each day for six consecutive days prior to the abdominal X-ray days. Participants were to ingest a total amount of 120 markers during the run-in period (day -6 to -1) and another 120 markers during the on-treatment period (day 8 to 13). Marker counts were identified by a radiologist who remained blinded to treatment assignment. CTTs were calculated according to the distribution of the ROMs in different segments – ascending colon, transverse colon and distal to the splenic flexure – of the bowel and summed to yield a total marker count. CTT was calculated using the classic film estimate as described in the following formula:

$$CTT = n_i \times (t/N)$$

where n_i is the number of ROMs observed on X-ray, t is the time between marker ingestions in hours, and N is the total number of ROMs ingested each day (Bouchoucha *et al.*, 1992; Bouchoucha *et al.*, 2006; Bouchoucha and Thomas, 2000). The ROMs were to be administered off-site, daily, at the same time each day, and the participants filmed themselves swallowing the markers to ensure compliance.

Defecation frequency and stool consistency

Participants recorded the number of defecations per day in the daily eDiary during the run-in period and during the two-week intervention period. Participants also recorded stool consistency from each defecation event. Stool consistency was rated in the eDiary using the BSS form (Lewis and Heaton, 1997).

PAC-SYM and PAC-QoL questionnaires

Participant assessment of constipation-related symptoms (PAC-SYM) and participant assessment of constipation-related quality of life (PAC-QoL) were evaluated at the clinic with PAC-SYM (Standard Version 2.0-Sd, 12-item) and PAC-QoL (Standard Version 2.0-Sd, 28-item) questionnaires at baseline (day 0) and at the end of the intervention (day 14) on a 5-point Likert scale (from 0 to 4) (Frank *et al.*, 1999; Lembo *et al.*, 2010; Marquis *et al.*, 2005).

Faecal microbial analysis for multi-strain product recovery

Faecal samples were collected from the participants at baseline (within day -6 until 0) before IP consumption, and at the end of the intervention period (within day 8 until 14). DNA was extracted from 200 mg of faecal sample as previously described (Lehtinen *et al.*, 2018).

Samples were analysed by real-time quantitative polymerase chain reaction (qPCR) for NCFM, Bl-04 and Lpc-37 (qPCR assay details in supplemental materials and Supplementary Table S5). All quantification was based on comparison to standard curves of pure target culture DNA. To determine a positive sample the following criteria were used: any individual sample signal must fall on the standard curve (meaning quantifiable); in cases where both baseline and post intervention were positive, only an increase from baseline would be counted as positive; any sample had to be positive following the above criteria in 2 out 3 of the assays used. Only samples from participants who delivered a faecal sample at both baseline and post-intervention were analysed.

Statistical methods

Detailed description of statistical analyses are presented in the Supplementary Materials and methods. In brief, the primary analyses were performed for two different study populations: The intention-to-treat (ITT) population and per-protocol (PP) population. ITT was considered the primary efficacy analysis population. In case of missing data, last observation carried forward method be applied to conduct the statistical analysis for the ITT population.

The primary efficacy analysis for abdominal bloating followed the multivariate approach (ANCOVA) controlling for baseline on the average VAS score for the change from baseline (day -7 to 1) to treatment period (day 7 to 13).

The secondary efficacy analyses for digestive symptoms in addition to bloating, CTT, PAC-SYM, PAC-QoL and number of defecations followed the same multivariate approach (with discrete PAC variables also non-parametric analysis was conducted). In addition, the BSS scores were categorised as constipation (1,2); optimal (3,4,5); or diarrhoea (6,7), and were compared with a chi-square test for homogeneity. McNemar's test was used to analyse how many participants moved between the categories. Overall product satisfaction was assessed at day 14 with a 5-point ordinal Likert scale (1 to 5) and evaluated using chi-square tests of homogeneity between the groups.

A post-hoc analysis was conducted to determine the differences in subgroups regarding VAS items (bloating, flatulence, abdominal pain, and burbling), CTT and bowel movement frequency. CTT and faecal microbial data (qPCR) were investigated further to define five subgroups for the post-hoc analysis: (1) ITT; (2) PP; (3) PP + CTT extremes excluded (PP + CTT); (4) PP + qPCR non-compliants excluded (PP + qPCR); (5) PP + CTT extremes excluded + qPCR non-compliants excluded (PP + CTT + qPCR). A few participants were found to have abnormal CTT values (less than 24 hours or more than 100 hours during the treatment period), thus these participants were excluded from the subgroup 3. The qPCR results were used to ensure that the IP were properly consumed, thus additional subgroups 4 and 5 were formed.

Repeated measurement models (for VAS values), Wilcoxon two-sample test (for CTT and AUCs describing the changes in VAS items) and generalised linear models (time to significant relief, bowel movement frequency) were used in the post-hoc analyses.

3. Results

Participants

In total, 264 participants were screened and 263 were enrolled into the study after receiving their voluntarily signed informed consent. A total of 156 of the randomised participants were treated with IP (78 with active and 78 with placebo treatment) and included in the safety analysis population. The ITT and PP populations comprised of 156 and 102 participants, respectively. Figure 2 shows the distribution of participants, including entries and withdrawals from this study in a CONSORT flow diagram. All 156 participants in the ITT population met the eligibility criteria at screening and there were no missing data for these criteria. Participants were assigned to a treatment group based on the administered treatment. The randomisation code was not broken for any of the participants during the study. Unblinding of the randomisation code occurred after database lock.

Safety results

There were no AEs reported that were probably or definitely related to the active or placebo treatment. The summary of AEs is presented in Supplementary Table S2. There were no clinical safety concerns with study product, no events leading to death and no SAEs were reported in this study. IP consumption was discontinued for two participants because of the administration of antibiotic treatment following an AE: one participant in the active group had a tooth abscess and one participant in the active group had *Herpes simplex*. There were no clinically significant abnormal vital signs, physical findings or other observations related to study.

Subjective assessment of digestive symptoms

No statistically significant differences were observed between the two treatment groups in the primary efficacy analysis for self-reported bloating. The mean VAS score for abdominal bloating at baseline in the ITT population was similar (P=0.925) in the two treatment groups and the mean change from baseline to treatment was also similar (P=0.965) in the two groups (Supplementary Table S3).

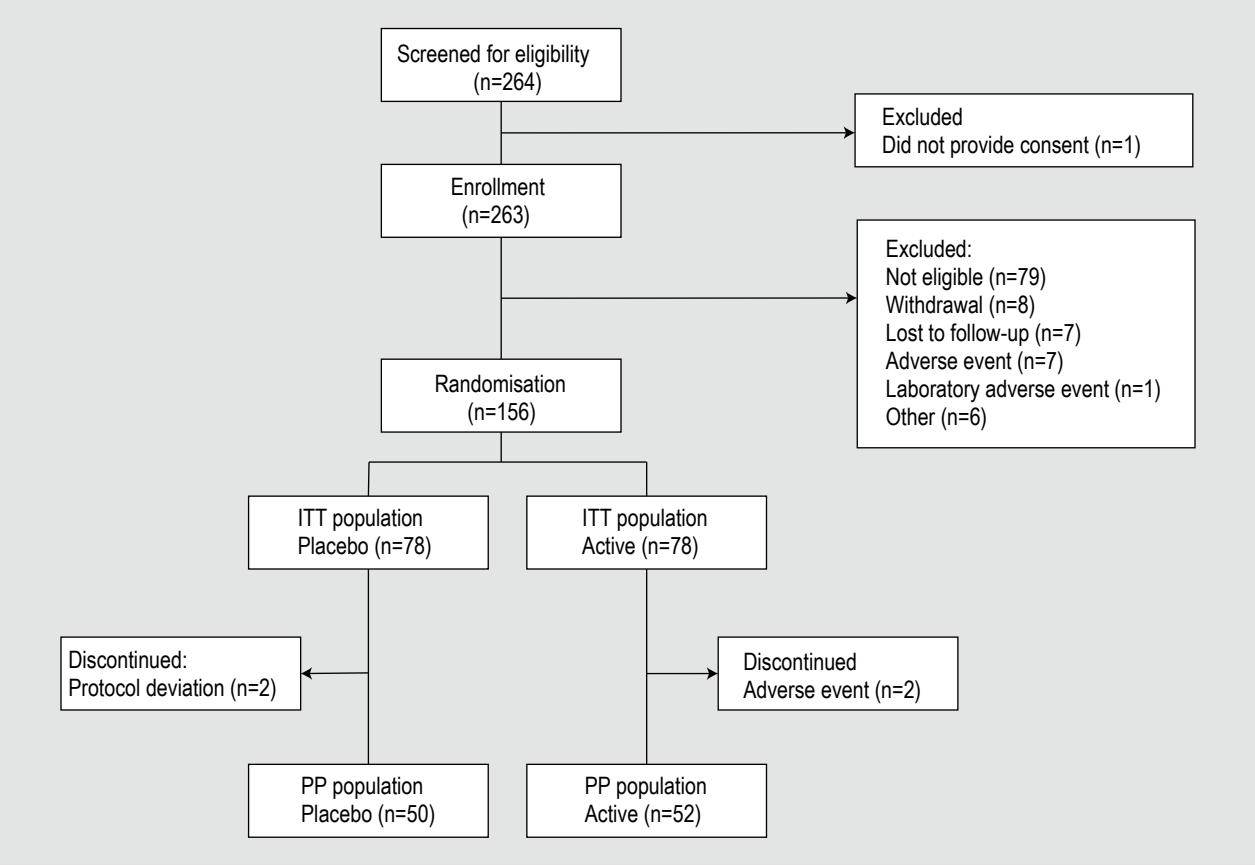

Figure 2. Disposition of participants in a CONSORT flow diagram. The demographic and physical characteristics of the two treatment groups in the intention-to-treat (ITT) population were similar (Supplementary Table S1), and there were no notable differences in demographics between the ITT and the per-protocol (PP) population. The physical activity level in both groups did not change during the study according to the international physical activity questionnaire results, and there were almost equal number of subjects within each activity level at baseline and post-intervention.

This change from baseline to treatment was negative, showing that the symptom scores were decreasing. In the PP population, the mean average bloating VAS scores at baseline demonstrated a slightly larger, although not statistically significant (P=0.366), difference than in the ITT population, and the mean change from baseline to treatment was similar (P=0.986) in the two groups (Supplementary Table S3). The treatment difference for change scores (controlling for baseline) was not statistically significant in ITT (P=0.987) or PP (P=0.766) populations (Supplementary Table S3). The secondary efficacy analysis of flatulence, abdominal pain, and burbling, showed no statistically significant treatment effects in the ITT nor PP populations (Supplementary Table S3). The results of the cohort analysis were consistent with the main analysis.

Post-hoc analysis of subjective digestive symptoms, AUC values, were investigated. Improvements on the VAS scale have negative values, but for clarity were inverted to be positive as AUC values. The placebo and active groups did not differ in bloating, abdominal pain or burbling in any of the subgroups on day 7 and 14. However, there was a statistically significant difference in flatulence in ITT, PP, and PP + qPCR subgroups on day 7 (P-values 0.0313, 0.0253 and 0.0222, respectively) and day 14 (P-values 0.0116, 0.0102 and 0.0090, respectively) (Figure 3). The difference was in favour of the active group resulting in larger positive AUC values, i.e. lower flatulence VAS values, than in the placebo group. There were statistically significant differences also in the mean of all VAS measurements in favour of the active group on day 7 (ITT, P=0.0425) and day 14 (ITT, P=0.0208; PP, P=0.0438). The repeated measurements analysis results were in line with the non-parametric AUC results.

Colonic transit time

Not all participants consumed 100% of radio-opaque markers within the given time-window, therefore adjustments were needed in the CTT calculations (Ibarra *et al.*, 2017). There was no statistically significant evidence of a treatment effect on CTT in the ITT or PP populations (Supplementary Table S3), and the results of the cohort

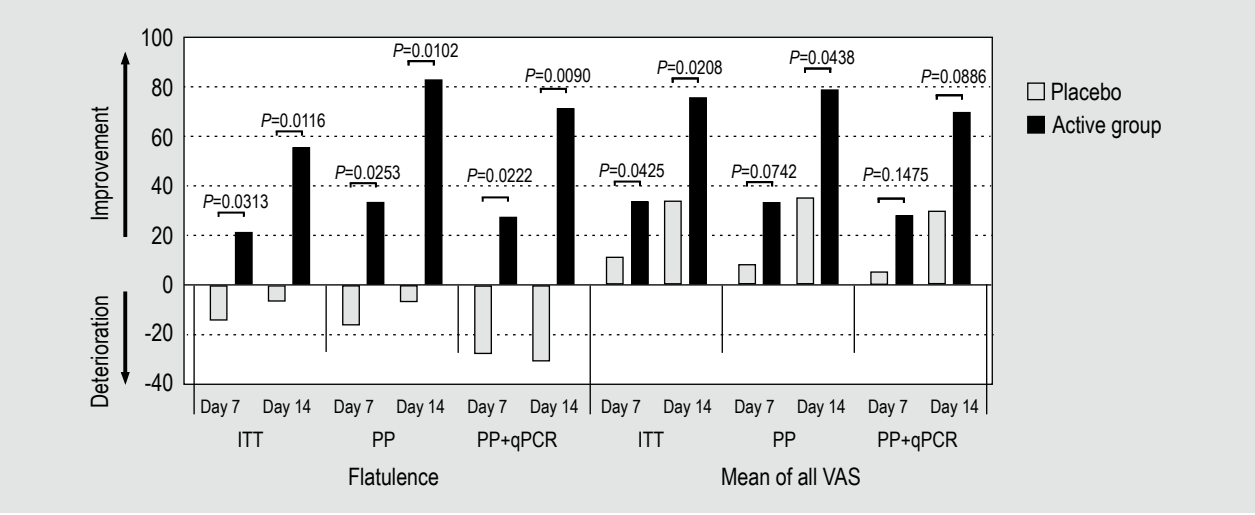

Figure 3. Average area under the curve (AUC) values for flatulence and mean of all digestive symptoms (baseline and treatment) in subgroups intention-to-treat (ITT), per-protocol (PP) and PP + qPCR non-compliants excluded (PP + qPCR) (day 7 and 14). Subjects showing improvement on the visual analogue scale (VAS) have negative values, which were inverted for clarity as AUC values.

analysis were consistent with the main analysis. At baseline, all groups had CTTs in line with other studies looking at constipated subjects (Miller *et al.*, 2017c) (Supplementary Table S3). The post-hoc analysis of CTT did not reveal any statistically significant differences between treatment groups, although in all subgroups the transit times were slightly shorter in the active group than in the placebo group on day 14 (Supplementary Figure S1).

Defecation frequency and stool consistency

Primary analysis showed no statistically significant evidence for treatment effect on defecation frequency in the ITT or PP populations (Supplementary Table S3) and the cohort analyses were consistent with the main analysis. According to the primary analysis, there was no statistically significant difference between the number of participants in each stool consistency category (constipation, optimal and diarrhoea) from baseline to treatment or from run-in to on-treatment (Supplementary Table S3). There were no significant differences between treatment groups in the number of participants transitioning between categories. The post-hoc analysis of bowel movement frequency from adjusted analysis revealed statistically significant difference (P=0.0453) between treatment groups in PP + CTT + qPCR subgroup favouring active group over placebo group. Bowel movement frequency was also explored post-hoc in connection with stool consistency, using event count before treatment and in both treatment weeks. This analysis showed that in both treatment groups, the consistency of stool was improved from constipation to optimal category. However, there was no difference between the treatment groups.

PAC-SYM and PAC-QoL questionnaires

There was no statistically significant evidence of a treatment effect on PAC-SYM or PAC-QoL questionnaires in the ITT population (Supplementary Table S3). The results for the PP population were consistent with those for the ITT population and the results of the cohort analysis were consistent with the main analysis.

Overall product satisfaction

There was no statistically significant difference between the product rating in the two treatment groups in the ITT population (Supplementary Table S3). The results for the PP population were consistent with those for the ITT population and the results of the cohort analysis were consistent with the main analysis.

Faecal recovery

Faecal microbial member abundance was analysed by qPCR as an exploratory parameter for determining probiotic recovery. A total of 264 samples from 132 participants (64 in the active group, 68 in the placebo group) with baseline and post-intervention timepoints were measured. Based on the detection of NCFM, Bl-04 and Lpc-37 in faeces, 71.7% of the active group were positive – according to the pre-defined criteria – and 93.5% of the placebo group were negative (Figure 4, Supplementary Table S4). Overall, the study had good probiotic recovery based on microbial qPCR analysis.

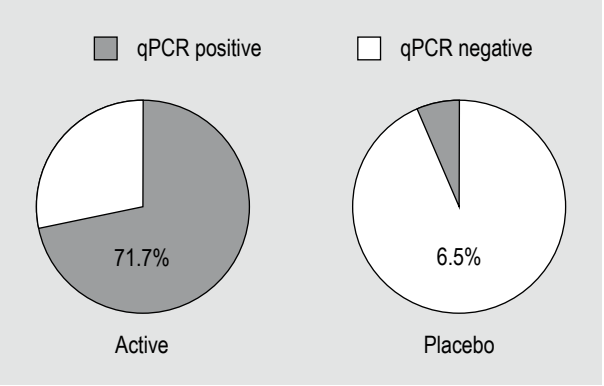

Figure 4. Multi-strain product recovery analysed by quantitative polymerase chain reaction (qPCR).

4. Discussion

Given the promising clinical effects of probiotics on gastrointestinal health to date, the objective of this clinical study was to evaluate the efficacy and safety of the two-week supplementation of a probiotic blend on bloating, CTT and other gastrointestinal symptoms in adults with self-reported bloating and constipation. The study had a randomised, double-blind, placebo-controlled design to minimise bias when comparing safety and efficacy data between the treatments. The strains NCFM, Lpc-37, Bl-04, Bi-07, and HN019 have previously been tested in clinical trials with efficacy shown in the digestive and immune health related areas, and are currently commercialised worldwide as dietary supplements. The dosages used in this trial have been proven safe for human consumption and in the clinical trials conducted with these strains, there have been no SAEs related to the IP or the trial procedures. No IP-related AEs or SAEs were reported in this study either, confirming the previously reported safety of these lactobacilli and bifidobacterial strains. In addition, there were no clinically significant abnormalities in vital signs or physical findings related to safety of the study product.

The randomisation of the study population was successful, and there were no notable differences in the baseline characteristics between the active and placebo group. FFQ was assessed only at the beginning of the trial to evaluate the fulfilment of the inclusion criteria. Since the management of constipation-related symptoms is often focused on lifestyle and diet, these variables could have been evaluated further. Regarding physical activity, a component possibly affecting digestive discomfort and transit time, there were no observed differences between the two study populations. Smoking status can affect digestive symptoms and especially quitting smoking has been reported to cause constipation (Hajek *et al.*, 2003). More current smokers were reported in the active group than in the placebo group. However, the number of ex-smokers was similar between groups. The primary results were similar in ITT and PP populations, even though a considerably high number of participants were excluded from PP population due to screening failures, withdrawals, and major protocol deviations. The observed results for product recovery in faeces by qPCR are in accordance for probiotic trials (Lehtinen *et al.*, 2018). This however, to the best of our knowledge, is the first multi-strain probiotic recovery reported in humans.

In primary analysis, the results for active and placebo were similar in this study. Our study population was not expressing as severe bloating as would have been expected from previously reported studies with constipated populations, where the pooled bloating severity was 3.0 (Fateh *et al.*, 2011; Waitzberg *et al.*, 2013). The inclusion limit for self-reported bloating was set to at least two bloating events per week, and the baseline value for bloating severity in our study population was 1.96. This may partly explain why we did not observe any significant improvement in the bloating severity in this trial, i.e. the study population was not bloated enough to allow for a substantial improvement. The effects of probiotics on bowel movement frequency are reported to be greater in studies where functional constipation was diagnosed using Rome III, compared to non-Rome diagnosis techniques (Miller *et al.*, 2017c). Also, in our study, we based the inclusion criteria to functional constipation or IBS-C by Rome III and verified the fulfilment of these criteria from the participants' self-assessment. Besides Rome III, other criteria, such as 1-3 bowel movements per week and stool consistency 1-2 on the BSS for most (≥50%) spontaneously passed stools, were selected in order to narrow down the population to those suffering from functional constipation and slow transit time. Miller *et al.* (2017b) reported pooled estimates for normative values in constipated population for stool frequency (2.7 weekly stools) and form (2.4 in BSS) according to Rome III criteria. The normative value for pooled CTT (58 hours) has also been reported by Miller *et al.* (2017a) in a systematic review and meta-analysis. CTT differ from person to person; on average, the CTT for a healthy individual is between 30-40 hours on average but can range to 70 hours (Bautista Casasnovas *et al.*, 1991; Kim and Rhee, 2012; Metcalf *et al.*, 1987). The newly published method for correction for non-compliance when determining CTT (Ibarra *et al.*, 2017) was implemented in this study, which added more accuracy in this evaluation. In the light of these expected normative baseline values, our ITT population – with 2.4 bowel movements per week, stool form 2.0 in BSS, and 66 hours' transit time – was suffering from functional constipation, albeit we did not observe significant changes in any of these outcomes.

A high placebo effect and the relatively short duration of the study could have contributed to the null primary analysis results. A strong placebo effect is common in many probiotic studies, especially in those involving

participants' self-evaluation of their symptom severity. Interestingly, there are other studies reporting beneficial effects of probiotics, especially HN019, already after two to four weeks' consumption. A study by Waller *et al.* (2011) reported significant improvements in CTT and frequency of functional GI symptoms already in two weeks in adult population diagnosed with constipation (Waller *et al.*, 2011). A more recent 28-day intervention with HN019 did not reveal any clear relief in transit time, but instead a physiologically relevant increase in weekly bowel movement frequency was observed in those participants who had fewer than three bowel movements per week initially (Ibarra *et al.*, 2018). Moreover, there seems to be evidence that longer interventions (60-105 days) may be required to determine the effects of probiotics on constipation symptoms (Riezzo *et al.*, 2018). The study by Waller *et al.* (2011) indicated that daily supplementation with HN019 for 14 days could decrease CTT dose-dependently. With respect to our study, a similar effect was not seen with a five-probiotic strain combination in two weeks, even though the total dose used was slightly higher than in the reference studies; 10^{10} cfu/day of HN019 and a total count of 2.75×10^{10}. Waller and co-workers (2011) used two dosages of HN019: 1.72×10^{10} cfu/day and 1.8×10^{9} cfu/day, while Ibarra *et al.* (2018) reported 10^{9} and 10^{10} cfu/day.

The additional post-hoc analysis revealed some differences between the probiotic and placebo treatments. A statistically significant difference between treatment groups in the severity of flatulence, measured with AUC, was noted on both study weeks in ITT and PP populations and in the PP subgroup, from which the qPCR non-compliants were removed. Moreover, the means of all the VAS scores also favoured the active group, indicating a possible trend toward symptom improvement. However, the differences of the means are only approximately 4-5% of the whole range of AUCs.

There were no differences found in CTT in any of the subgroups. However, there was some statistical evidence of greater increase in bowel movement frequency for active probiotic treatment in PP + CTT + qPCR subgroup, but the confidence interval was rather wide. Thus, one must be cautious when drawing conclusions of the effect of the study product to bowel movement frequency. While investigating the digestive symptoms post-hoc, one must remember that AUCs behave somewhat differently than the absolute symptom scores, as it is a cumulative analysis. Nonetheless, the post-hoc analysis revealed statistically significant differences in flatulence and mean VAS of all symptoms in the ITT and PP populations. Significant relief in flatulence was also observed in the PP subgroup from which the qPCR non-compliants were removed. This subgroup was the most compliant to the product consumption in terms of product recovery. Using the mean VAS besides the individual symptoms seems to be a valid addition providing information on symptoms' evolution on a general level.

In conclusion, although there were no differences in the primary analysis, the combination of five tested lactobacilli and bifidobacteria strains was well tolerated and safe to consume. In addition, the two-week consumption of this probiotic blend seemed to improve the self-reported digestive symptoms, especially flatulence, in adults with self-reported bloating and constipation.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2018.0163.

Supplementary Materials and methods
- Ethics and good clinical practices
- Exclusion criteria
- Investigational product
- qPCR assays
- Statistical methods

Table S1. Demographic and baseline characteristics of the intention-to-treat population.

Table S2. Summary of adverse events.

Table S3. Mean values of primary and secondary outcomes in intention-to treat and per-protocol populations.

Table S4. Faecal recovery qPCR results, shown as qualitatively positive by assay and, the criteria of being positive for 2 out of 3 or 3 out of 3 of the assays concurrently.

Table S5. Primer sequences and annealing temperatures.

Figure S1. eDiary average response curves in different subgroups: ITT, PP, PP + CTT, PP + qPCR, and PP + CTT + qPCR.

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Conflict of interest

DuPont Nutrition and Health and RenewLife (US, now Clorox) sponsored the study. The bacterial strains NCFM, Lpc-37, Bl-04, Bi-07, and HN019 are commercially marketed worldwide as dietary supplements by DuPont. KA, NY, AL, SJ and ACO were employed by DuPont at the time of the study. The authors declare no other conflict of interest.

References

Bautista Casasnovas, A., Varela Cives, R., Villanueva Jeremias, A., Castro-Gago, M., Cadranel, S. and Tojo Sierra, R., 1991. Measurement of colonic transit time in children. Journal of Pediatric Gastroenterology and Nutrition 13: 42-45.

Belsey, J., Greenfield, S., Candy, D. and Geraint, M., 2010. Systematic review: impact of constipation on quality of life in adults and children. Alimentary Pharmacology and Therapeutics 31: 938-949.

Bettler, J., Mitchell, D.K. and Kullen, M.J., 2006. Administration of *Bifidobacterium lactis* with fructo-oligosaccharide to toddlers is safe and results in transient colonization. International Journal of Probiotics and Prebiotics 1 (3-4).

Bingham, S.A., Welch, A.A., McTaggart, A., Mulligan, A.A., Runswick, S.A., Luben, R., Oakes, S., Khaw, K.T., Wareham, N. and Day, N.E., 2001. Nutritional methods in the European prospective investigation of cancer in Norfolk. Public Health Nutrition 4: 847-858.

Bouchoucha, M., Devroede, G., Arhan, P., Strom, B., Weber, J., Cugnenc, P.H., Denis, P. and Barbier, J.P., 1992. What is the meaning of colorectal transit time measurement? Diseases of the Colon and Rectum 35: 773-782.

Bouchoucha, M., Devroede, G., Faye, A., Le Toumelin, P., Arhan, P. and Arsac, M., 2006. Colonic responce to food in constipation. International Journal of Colorectal Disease 21: 826-833.

Bouchoucha, M. and Thomas, S.R., 2000. Error analysis of classic colonic transit time estimates. American Journal of Physiology-Gastrointestinal and Liver Physiology 279: G520-527.

Dekker, J.W., Wickens, K., Black, P.N., Stanley, T.V., Mitchell, E.A., Fitzharris, P., Tannock, G.W., Purdie, G. and Crane, J., 2009. Safety aspects of probiotic bacterial strains *Lactobacillus rhamnosus* HN001 and *Bifidobacterium animalis* subsp. lactis HN019 in human infants aged 0-2 years. International Dairy Journal 19: 149-154.

Drossman, D.A., 2006. The functional gastrointestinal disorders and the Rome III process. Gastroenterology 130: 1377-1390.

Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO), 2001. Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria. Available at: http://tinyurl.com/8bccc3r.

Fateh, R., Iravani, S., Frootan, M., Rasouli, M.R. and Saadat, S., 2011. Synbiotic preparation in men suffering from functional constipation: a randomised controlled trial. Swiss Medical Weekly 141: w13239.

Favretto, D.C., Pontin, B. and Moreira, T.R., 2013. Effect of the consumption of a cheese enriched with probiotic organisms (*Bifidobacterium lactis* BI-07) in improving symptoms of constipation. Arquivos de Gastroenterologia 50: 196-201.

Francis, C.Y., Morris, J. and Whorwell, P.J., 1997. The irritable bowel severity scoring system: a simple method of monitoring irritable bowel syndrome and its progress. Alimentary Pharmacology and Therapeutics 11: 395-402.

Frank, L., Kleinman, L., Farup, C., Taylor, L. and Miner, P., 1999. Psychometric validation of a constipation symptom assessment questionnaire. Scandinavian Journal of Gastroenterology 34: 870-877.

Hagstromer, M., Oja, P. and Sjostrom, M., 2006. The International Physical Activity Questionnaire (IPAQ): a study of concurrent and construct validity. Public Health Nutrition 9: 755-762.

Hajek, P., Gillison, F. and McRobbie, H., 2003. Stopping smoking can cause constipation. Addiction 98: 1563-1567.

Harris, L.A., 2005. Prevalence and ramifications of chronic constipation. Managed Care Interface 18: 23-30.

Heidelbaugh, J.J., Stelwagon, M., Miller, S.A., Shea, E.P. and Chey, W.D., 2015. The spectrum of constipation-predominant irritable bowel syndrome and chronic idiopathic constipation: US survey assessing symptoms, care seeking, and disease burden. American Journal of Gastroenterology 110: 580-587.

Hemalatha, R., Ouwehand, A.C., Forssten, S.D., Geddan, J.J.B., Mamidi, R.S., Bhaskar, V. and Radhakrishna, K.V., 2014. Community-based randomized double blind controlled trial of *Lactobacillus paracasei* and *Bifidobacterium lactis* on reducing risk for diarrhea and fever in preschool children in an urban slum in India. European Journal of Nutrition and Food Safety 4: 325-341.

Higgins, P.D. and Johanson, J.F., 2004. Epidemiology of constipation in North America: a systematic review. American Journal of Gastroenterology 99: 750-759.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., Calder, P.C. and Sanders, M.E., 2014. Expert consensus document: the International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology and Hepatology 11: 506-514.

Ibarra, A., Latreille-Barbier, M., Donazzolo, Y., Pelletier, X. and Ouwehand, A.C., 2018. Effects of 28-day *Bifidobacterium animalis* subsp. *lactis* HN019 supplementation on colonic transit time and gastrointestinal symptoms in adults with functional constipation: a double-blind, randomized, placebo-controlled, and dose-ranging trial. Gut Microbes 9: 236-251.

Ibarra, A., Olli, K. and Ouwehand, A.C., 2017. Correcting for non-compliance when determining colonic transit time with radio-opaque markers. World Journal of Gastroenterology 23: 740-742.

International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), 1996. Guideline for Good Clinical Practice. ICH Harmonised Tripartite Guideline. E6(R1). Current Step 4 version, dated 10 June 1996 (including the Post Step 4 corrections). Available at: http://apps.who.int/medicinedocs/en/m/abstract/Js22154en/.

Iovino, P., Bucci, C., Tremolaterra, F., Santonicola, A. and Chiarioni, G., 2014. Bloating and functional gastro-intestinal disorders: where are we and where are we going? World Journal of Gastroenterology 20: 14407-14419.

Kim, E.R. and Rhee, P.L., 2012. How to interpret a functional or motility test - colon transit study. Journal of Neurogastroenterology and Motililty 18: 94-99.

Lehtinen, M.J., Hibberd, A.A., Mannikko, S., Yeung, N., Kauko, T., Forssten, S., Lehtoranta, L., Lahtinen, S.J., Stahl, B., Lyra, A. and Turner, R.B., 2018. Nasal microbiota clusters associate with inflammatory response, viral load, and symptom severity in experimental rhinovirus challenge. Scientific Reports 8: 11411.

Lembo, A.J., Kurtz, C.B., Macdougall, J.E., Lavins, B.J., Currie, M.G., Fitch, D.A., Jeglinski, B.I. and Johnston, J.M., 2010. Efficacy of linaclotide for patients with chronic constipation. Gastroenterology 138: 886-895.

Lewis, S.J. and Heaton, K.W., 1997. Stool form scale as a useful guide to intestinal transit time. Scandinavian Journal of Gastroenterology 32: 920-924.

Locke 3rd, G.R., Pemberton, J.H. and Phillips, S.F., 2000. AGA technical review on constipation. American Gastroenterological Association. Gastroenterology 119: 1766-1778.

Longstreth, G.F., Thompson, W.G., Chey, W.D., Houghton, L.A., Mearin, F. and Spiller, R.C., 2006. Functional bowel disorders. Gastroenterology 130: 1480-1491.

Magro, D.O., De Oliveira, L.M., Bernasconi, I., De Souza Ruela, S., Credidio, L., Barcelos, I.K., Leal, R.F., Lourdes Setsuko Ayrizono, M., Fagundes, J.J., De B Teixeira, L., Ouwehand, A.C. and Coy, C.S., 2014. Effect of yogurt containing polydextrose, *Lactobacillus acidophilus* NCFM and *Bifidobacterium lactis* HN019: a randomized, double-blind, controlled study in chronic constipation. Nutrition Journal 13: 75.

Marquis, P., De la Loge, C., Dubois, D., McDermott, A. and Chassany, O., 2005. Development and validation of the patient assessment of constipation quality of life questionnaire. Scandinavian Journal of Gastroenterology 40: 540-551.

Metcalf, A.M., Phillips, S.F., Zinsmeister, A.R., MacCarty, R.L., Beart, R.W. and Wolff, B.G., 1987. Simplified assessment of segmental colonic transit. Gastroenterology 92: 40-47.

Miller, L.E., Ibarra, A. and Ouwehand, A.C., 2017a. Normative values for colonic transit time and patient assessment of constipation in adults with functional constipation: systematic review with meta-analysis. Clinical Medicine Insights: Gastroenterology 10: 1179552217729343.

Miller, L.E., Ibarra, A., Ouwehand, A.C. and Zimmermann, A.K., 2017b. Normative values for stool frequency and form using Rome III diagnostic criteria for functional constipation in adults: systematic review with meta-analysis. Annals of Gastroenterology 30: 161-167.

Miller, L.E., Ouwehand, A.C. and Ibarra, A., 2017c. Effects of probiotic-containing products on stool frequency and intestinal transit in constipated adults: systematic review and meta-analysis of randomized controlled trials. Annals of Gastroenterology 30: 629-639.

Miller, L.E., Zimmermann, A.K. and Ouwehand, A.C., 2016. Contemporary meta-analysis of short-term probiotic consumption on gastrointestinal transit. World Journal of Gastroenterology 22: 5122-5131.

Ouwehand, A.C., DongLian, C., Weijian, X., Stewart, M., Ni, J., Stewart, T. and Miller, L.E., 2014. Probiotics reduce symptoms of antibiotic use in a hospital setting: a randomized dose response study. Vaccine 32: 458-463.

Raahave, D., Christensen, E., Loud, F.B. and Knudsen, L.L., 2009. Correlation of bowel symptoms with colonic transit, length, and faecal load in functional faecal retention. Danish Medical Bulletin 56: 83-88.

Riezzo, G., Orlando, A., D'Attoma, B., Linsalata, M., Martulli, M. and Russo, F., 2018. Randomised double blind placebo controlled trial on *Lactobacillus reuteri* DSM 17938: improvement in symptoms and bowel habit in functional constipation. Beneficial Microbes 9: 51-60.

Ringel-Kulka, T., Goldsmith, J.R., Carroll, I.M., Barros, S.P., Palsson, O., Jobin, C. and Ringel, Y., 2014. *Lactobacillus acidophilus* NCFM affects colonic mucosal opioid receptor expression in patients with functional abdominal pain – a randomised clinical study. Alimentary Pharmacology and Therapeutics 40: 200-207.

Rousseaux, C., Thuru, X., Gelot, A., Barnich, N., Neut, C., Dubuquoy, L., Dubuquoy, C., Merour, E., Geboes, K., Chamaillard, M., Ouwehand, A., Leyer, G., Carcano, D., Colombel, J.F., Ardid, D. and Desreumaux, P., 2007. *Lactobacillus acidophilus* modulates intestinal pain and induces opioid and cannabinoid receptors. Nature Medicine 13: 35-37.

Turroni, F., Ventura, M., Butto, L.F., Duranti, S., O'Toole, P.W., Motherway, M.O. and Van Sinderen, D., 2014. Molecular dialogue between the human gut microbiota and the host: a *Lactobacillus* and *Bifidobacterium* perspective. Cellular and Molecular Life Sciences 71: 183-203.

Waitzberg, D.L., Logullo, L.C., Bittencourt, A.F., Torrinhas, R.S., Shiroma, G.M., Paulino, N.P. and Teixeira-da-Silva, M.L., 2013. Effect of synbiotic in constipated adult women – a randomized, double-blind, placebo-controlled study of clinical response. Clinical Nutrition 32: 27-33.

Waller, P.A., Gopal, P.K., Leyer, G.J., Ouwehand, A.C., Reifer, C., Stewart, M.E. and Miller, L.E., 2011. Dose-response effect of *Bifidobacterium lactis* HN019 on whole gut transit time and functional gastrointestinal symptoms in adults. Scandinavian Journal of Gastroenterology 46: 1057-1064.

World Medical Association (WMA), 2013. World medical association declaration of helsinki: ethical principles for medical research involving human subjects. World Medical Association Declaration of Helsinki, 7th edition. JAMA 310: 2191-2194. DOI: https://doi.org/10.1001/jama.2013.281053