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Metabolic adaptation of colonic microbiota to galactooligosaccharides: a proof-of-concept-study

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SUMMARY

Background
Prebiotics have been shown to reduce abdominal symptoms in patients with functional gut disorders, despite that they are fermented by colonic bacteria and may induce gas-related symptoms.

Aim
To investigate changes in the metabolic activity of gut microbiota induced by a recognised prebiotic.

Methods
Healthy subjects (n = 20) were given a prebiotic (2.8 g/day HOST-G904, HOST Therabiomics, Jersey, Channel Islands) for 3 weeks. During 3-day periods immediately before, at the beginning and at the end of the administration subjects were put on a standard diet (low fibre diet supplemented with one portion of high fibre foods) and the following outcomes were measured: (i) number of daytime gas evacuations for 2 days by means of an event marker; (ii) volume of gas evacuated via a rectal tube during 4 h after a test meal; and (iii) microbiota composition by faecal Illumina MiSeq sequencing.

Results
At the beginning of administration, HOST-G904 significantly increased the number of daily anal gas evacuations (18 ± 2 vs. 12 ± 1 pre-administration; $P < 0.001$) and the volume of gas evacuated after the test meal (236 ± 23 mL vs. 160 ± 17 mL pre-administration; $P = 0.006$). However, after 3 weeks of administration, these effects diminished (11 ± 2 daily evacuations, 169 ± 23 mL gas evacuation). At day 21, relative abundance of butyrate producers (Lachnospiraceae) correlated inversely with the volume of gas evacuated ($r = -0.52$; $P = 0.02$).

Conclusion
The availability of substrates induces an adaptation of the colonic microbiota activity in bacterial metabolism, which produces less gas and associated issues. Clinical trials.gov NCT02618239.

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INTRODUCTION

Prebiotics, by definition, induce beneficial effects by selectively influencing colonic microbiota.[1] They serve as selective substrates for microbiota gut metabolism.[2]

The effect of fibre on functional intestinal symptoms is controversial.[3] We recently showed that a diet rich in non-absorbable, fermentable residues in the short term increased intestinal gas production and induced digestive symptoms, such as flatulence, abdominal bloating and distension,[4] whereas a low-residue diet improved symptoms in patients with abdominal bloating and flatulence.[5] Good evidence of the clinical benefits of reducing fermentable foodstuffs has been provided by a series of studies using diets low in fermentable oligosaccharides, monosaccharides, disaccharides and polyols (FODMAP).[6–8]

In contrast to the potential effect of low-residue diets on symptoms, some specific prebiotics, despite being fermented by microbiota, have been shown to improve these types of symptoms. Specifically, a clinical trial with the prebiotic HOST-G904 demonstrated a clinical benefit in patients with irritable bowel syndrome.[9] A very elegant controlled trial in healthy university students showed that around the time of final exams, stress was associated with diarrhoea, indigestion and abdominal pain, and galactooligosaccharide supplementation reduced this stress-induced gastrointestinal dysfunction.[10.]

We hypothesised that prebiotic administration initially activates the fermentative metabolism of colonic microbiota, increasing gas production, and that this early effect is later followed by an adaptation of the microbiota with a reduction in net gas production. Our aim was to assess the effect of HOST-G904,[9] on microbiota gas production at initial exposure and then following continuous administration for a period of potential adaptation. To this end, we designed a proof-of-concept study in healthy subjects.

MATERIALS AND METHODS

Participants

Twenty-six healthy subjects without gastrointestinal symptoms or history of gastrointestinal disorders participated in the study: 20 subjects participated in the main study, and six subjects, as a control group, in an ancillary study (Table 1). All participants were instructed to fill out a clinical questionnaire based on Rome III criteria to rule out functional gastrointestinal disorders (no symptom ≥2 on a 0–10 scale) and to confirm normal bowel habits. This questionnaire has been previously shown to discriminate patients from healthy subjects.[4, 5, 11–14] Antibiotic, but not pre- or probiotic consumption during the previous 2 month was an exclusion criterium. Subjects gave written informed consent to participate in the study. The protocol, including the external control study, was approved by the Institutional Review Board of University Hospital Vall d'Hebron and was registered with ClinicalTrials.Gov [NCT02618239].

Table 1 | Demographic data

	Main study	Ancillary study
Age range, year	18–54	25–35
Women/men, *n*	12/8	5/1
BMI range, kg/m^2	19–26	22–27
Bowel movements/week, mean ± S.E.	8 ± 1	8.5 ± 1.2
Bristol score, mean ± S.E.	3.6 ± 0.2	3.8 ± 0.3

Experimental design

Participants consumed a prebiotic (2.8 g/day HOST-G904, HOST Therabiomics, Jersey, Channel Islands) for 3 week. For three 3-day periods, pre-administration (day −2 to 0), at the early administration period (day 1–3) and the late administration period (day 19–21), different outcomes (see below) were measured (evaluation periods).

Diet

During the study participants consumed their habitual diet except during the evaluation periods when the diet was standardised, as follows. During the evaluation periods, subjects were instructed to consume a low fibre diet[5] restricted to the following foodstuffs: (i) meat, fish, fowl and eggs; (ii) salad; (iii) rice, pasta and bread; (iv) dairy products and (v) strained orange juice, tangerine, pears, apples and berries. This low-residue diet was complemented with one portion per day of the following: whole crackers, lentils, chickpeas, beans, peas, artichoke, Brussels' sprouts, banana, peach or prunes; the portion size of each specific foodstuff was adjusted to contain 12 g fibre. For the rest of the administration period, the participants consumed their usual diet. For the duration of the study, fermented dairy products and any tablets, pills or food supplements containing pre-or probiotics were not allowed (Figure 1). During the 3-day evaluation periods, participants were instructed to fill out a diary specifying the foods they consumed, to assess compliance with the diet and to calculate dietary intake.[15–17]

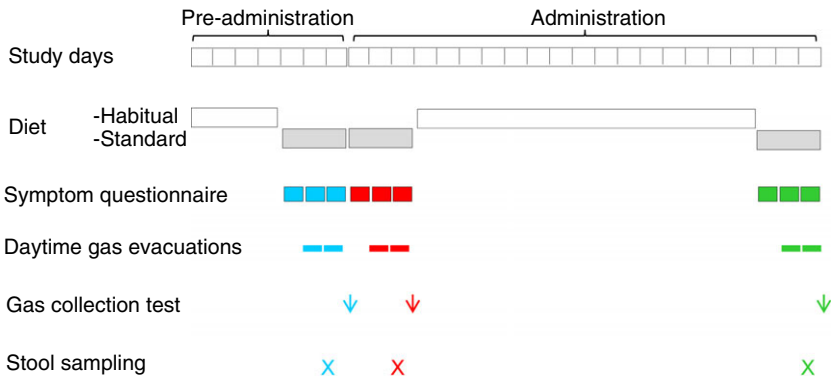


Figure 1 | Experimental design. All participants completed the main study (n = 20) and were included for analysis. Note the colour code: pre-administration in blue, early administration red, and late administration period green.

Outcomes

The following data were collected during the 3-day evaluation periods before, at the beginning and at the end of administration.

Daily symptom questionnaire. During the 3 day of each evaluation period, the participants were instructed to fill out daily questionnaires that included the following parameters: (i) subjective sensations of flatulence (defined as anal gas evacuation), abdominal bloating (pressure/fullness), abdominal distension (sensation of girth increment), borborygmi and abdominal discomfort/pain using 0–10 analogue scales; (ii) digestive well-being using a 10-point scale graded from +5 (extremely pleasant sensation/satisfaction) to −5 (extremely unpleasant sensation/dissatisfaction) and mood on similr scale graded from +5 (very positive) to −5 (very negative). This questionnaire has been previously used and was shown sensitive to detect effect of dietary interventions in different populations.[4, 5, 14, 18]

Number of anal gas evacuations. The number of anal gas evacuations was measured during the day using an event marker (Hand Tally Counter No 101, Digi Sport Instruments, Shanggiu, China). Participants were instructed to carry the event marker during the day and register each passage of anal gas. To facilitate compliance, anal gas evacuations were only registered during the last 2 day of each evaluation period. This method has been previously used with reproducible and consistent results[4, 5]; furthermore, studies measuring the number of gas evacuations by an event marker and continuously recording anal gas evacuations have shown a very good correlation ($r > 0.95$; $P < 0.05$).[19–22]

Microbiota composition. Faecal samples were collected on the last day of each evaluation period, i.e. immediately before and on the third and 21st day of administration (60 samples in total). After collection and homogenisation, the samples were immediately frozen by the participants in their home freezers at −20 °C and later brought to the laboratory in a freezer pack, where they were stored at −80 °C.

Genomic DNA extraction: A frozen aliquot (250 mg) of each sample was suspended in 250 μL of guanidine thiocyanate, 0.1 M Tris (pH 7.5), 40 μL of 10% N-lauroyl sarcosine and 500 μL 5% N-lauroyl sarcosine. DNA was extracted by mechanical disruption of microbial cells with beads, and recovery of nucleic acids from clear lysates was achieved by alcohol precipitation, as previously described.[23] An equivalent of 1 mg of each sample was used for DNA quantification using a NanoDrop ND-1000 Spectrophotometer (Nucliber, Madrid, Spain).

High-throughput DNA sequencing: For profiling the microbiome composition, the hyper-variable region (V4) of the bacterial and archaeal 16S rRNA gene was amplified by PCR. On the basis of our analysis performed using PrimerProspector software,[24] the V4 primer pairs used in this study were expected to amplify almost 100% of the bacterial and archaeal domains. The 5′ ends of the forward (V4F_515_19: 5′-GTGCCAGCAMGCCGCG GTAA-3′) and reverse (V4R_806_20: 5′-GGACTACCAG GGTATCTAAT-3′) primers targeting the 16S gene were tagged with specific sequences as follows: 5′-{AATGAT ACGGCGACCACCGAGATCTACACTATGGTAATTGT} {GTGCCAGCMGCCGCGGTAA}-3′ and 5′-{CAAGCAG AAGACGGCATACGAGAT} {Golay barcode} {AGTCA GTCAGCC} {GGACTACHVGGGTWTCTAAT}-3′. Multiplex identifiers, known as Golay codes, had 12 bases and were specified downstream of the reverse primer sequence (V4R_806_20).[25] Standard PCR (0.75 units of Taq polymerase (Roche, Barcelona, Spain) and 20 pmol/μL of the forward and reverse primers) was run in a Mastercycler gradient (Eppendorf, Madrid, Spain) at 94 °C for 3 min,

followed by 35 cycles of 94 °C for 45 s, 56 °C for 60 s, 72 °C for 90 s, and a final cycle of 72 °C for 10 min. Amplicons were purified using a QIAquick PCR Purification Kit (Qiagen, Barcelona, Spain), quantified using a NanoDrop ND-1000 Spectrophotometer (Nucliber, Madrid, Spain), and then pooled in equal concentrations. Pooled amplicons (2 nM) were then subjected to sequencing using Illumina MiSeq technology in the technical support unit of the Autonomous University of Barcelona (UAB, Spain) following standard Illumina platform protocols.

Sequence analysis: Sequences obtained from the 60 faecal samples after the sequencing step were analysed with QIIME (Quantitative Insights Into Microbial Ecology) 1.9.1[26] using an in-house script that performs upstream and downstream analyses. Low-quality raw sequences with a Phred score of less than 20 were removed from the analysis. Each read was assigned back to its corresponding sample during a demultiplexing step and barcodes were removed from the sequences. After filtering, we obtained a total of 2 460 589 high-quality sequences. The USEARCH (Ultra-fast Sequence Analysis)[27] tool was used to cluster similar sequences into Operational Taxonomic Units (OTUs) or taxa based on a 97% similarity and to remove chimeric sequences with the UCHIME (Ultra-fast Chimeric search) algorithm. From each of these OTUs, one representative sequence was selected and then aligned using PyNAST (Python Nearest Alignment Space Termination tool) against a Greengenes template alignment from the most recent version of the database (gg_13_8). Then, a taxonomical assignment step was performed using the basic local alignment search tool (BLAST) to map each representative sequence against a combined database encompassing the Greengenes and PATRIC (Pathosystems Ressource Integration Center) databases. A phylogenetic tree using the FastTree programme and an OTU table were built. To avoid false positive OTUs, we eliminated those that did not represent at least 0.2% of the sequences in at least two samples. The final OTU table was rarefied at 15396 sequence reads per sample. Rarefaction is used to overcome cases in which read counts were not similar between samples.

Quantification of Bifidobacterium*:* To assess *Bifidobacterium* genus quantification, the extracted genomic DNA was used to amplify the 16S rRNA gene by quantitative real-time PCR (qPCR) using the following specific primers Bifgenus_F: 5′-TGG CTC AGG ATG AAC GCT G-3′ and Bifgenus_R: 5′-TGA TAG GAC GCG ACC CCA T-3′ and TaqMan MGB probe (FAM™ dye-labeled): 5′-CAT CCG GCA TTA CCA-3′. To calibrate the qPCR reactions, we used calculated amounts of extracted DNA from three isolated *Bifidobacterium* species (*B. breve, B. longum and B. infantis*). Serial dilutions of the pooled DNA were amplified (copy number ranging from 25 to 2.5 × 106) to extrapolate the bifidobacterial number in each sample. The qPCR was performed with the 7500 Fast Real-Time PCR System (Applied Biosystems, Barcelona, Spain) using optical-grade 96-well plates. The PCR reaction was performed in a total volume of 25 μL using the TaqMan Universal PCR Master Mix (Applied Biosystems), containing 300 nM of each primer and 100 nM of MGB probe. The reaction conditions for amplification of DNA were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were performed in triplicate and mean values were calculated Data were analysed using Sequence Detection Software version 1.4, supplied by Applied Biosystems.

Response to a probe meal. The day following each evaluation period, participants reported to the laboratory after an overnight fast and the response to a probe meal was evaluated. The *probe meal* consisted of a ham omelet (100 g), 46 g of white bread, 10 g of butter, 25 g of jam and 200 mL of fruit juice (a 400 Kcal caloric content, 350 mL of total volume, 1.5 g of fibre). The first dose of HOST-G904 was administered after the first collection test; in the other two sets (early and late administration period) HOST-G904 was administered with the probe meal.

Anal gas evacuation: The volume of gas evacuated per anus was measured for 4 h after the probe meal, as previously described.[4, 28, 29] In brief, gas was collected using a rectal balloon catheter (20 F Foley catheter, Bard, Barcelona, Spain) connected via a gas-tight line to a barostat, and the volume was continuously recorded. The intrarectal balloon was inflated with 5 mL of water to prevent anal gas leaks.

Abdominal symptoms: Perception of abdominal sensations was measured every 30 min during the 4-h gas collection period using the same scales as described above: 0–10 scales for scoring abdominal bloating (pressure/fullness), abdominal distension (sensation of

girth increment), borborygmi and abdominal discomfort/pain; −5 to + 5 scales for scoring digestive well-being and mood.

Girth measurement: The method has been previously described.[30] Briefly, a non-stretch belt (48-mm wide) was placed over the umbilicus. The belt had a metric tape with marks at 1 mm intervals fixed over it. The overlapping ends of the belt were adjusted carefully by two elastic bands to ensure that the belt was in constant contact with the abdominal wall. Girth measurements down to the one-millimetre level were taken without manipulation by the investigator at 30-min intervals during the study. Previous studies validated the reproducibility of the measurements and the sensitivity of this method to consistently detect small variations in girth induced by various experimental conditions.[12, 30–33] Changes in girth during the infusion period were compared to the measurements during the basal period.

Ancillary study: external control group

In the control group (see Participants) HOST-G904 was administered at the same dose (2.8 g/day) for 2 weeks while participants consuming their usual diet (see Experimental design). The number of anal gas evacuations (see above) was measured during daytime before administration (day 0), and on day 2, 3, 5, 7, 12 and 15 during administration.

Statistical analysis

Microbiota analysis. The Shapiro–Wilk test was used to check the normality of the data, and pairwise comparisons were made between the study groups with the non-parametric Kruskal–Wallis one-way analysis of variance test, which compares means between groups. A false discovery rate (FDR) of corrected *P*-values was taken into account to consider the significance of the results.

Overall comparisons. The means (±S.E.) of the variables measured were calculated. The Kolmogorov–Smirnov test was used to check the normality of the data distribution. Parametric normally distributed data were compared by Student's *t*-test for paired or unpaired data; otherwise, the Wilcoxon signed-rank test was used for paired data and the Mann–Whitney *U* test for unpaired data. The association of parameters was analysed using linear regression analysis.

RESULTS

Study flow and dietary intake

All participants included in the study ($n = 26$) completed the protocols (main or ancillary study) and were included for analysis. Participants reported adherence to study instructions. Based on the diaries, dietary intake during each 3-day evaluation period was calculated, and mean daily intake during the three evaluation periods was similar (Table 2).

Symptoms and gas volume

Pre-administration. Before administration, participants tolerated the standardised diet (Table 2) without a significant perception of abdominal symptoms, except for a mild-sensation of flatulence. Interestingly, participants scored a positive sensation of digestive well-being and positive mood (Figure 2). Using the event marker, a mean of 12 ± 1 daytime anal gas evacuations was recorded (Figure 3). During the 4-h gas collection period after the probe meal, subjects evacuated 160 ± 17 mL of gas (Figure 4) without reporting a significant perception or abdominal distension (Figure 5); the scores of abdominal sensation, well-being and mood were similar to those recorded in diaries on previous day.

Early administration period. At the beginning of the HOST-G904 administration, no changes in abdominal sensations, well-being or mood were detected (Figure 2), but a clear effect on colonic gas production was observed. Indeed, on the standard diet (Table 2) the number of daytime gas evacuations increased by 39 ± 9%; up to 18 ± 2 daytime evacuations ($P < 0.001$ vs. pre-administration), and the effect was already present on the 2nd day of administration (Figure 3).

Table 2 | Daily dietary intake during evaluation periods

	Study periods: HOST-G904 administration		
	Before	Early phase	Late phase
GOS*, g	0.17 ± 0.04	0.19 ± 0.03	0.19 ± 0.03
Fructans, g	2.8 ± 0.3	2.9 ± 0.2	2.6 ± 0.2
Fibre, g	18.4 ± 0.4	18.4 ± 0.4	18.8 ± 0.4
Carbohydrates, g	292 ± 9	292 ± 9	286 ± 7
Lipids, g	47 ± 1	46 ± 1	48 ± 1
Proteins, g	86 ± 2	83 ± 2	86 ± 2

Data are means of 3 day in each evaluation period.

* Galacto-oligosaccharides.

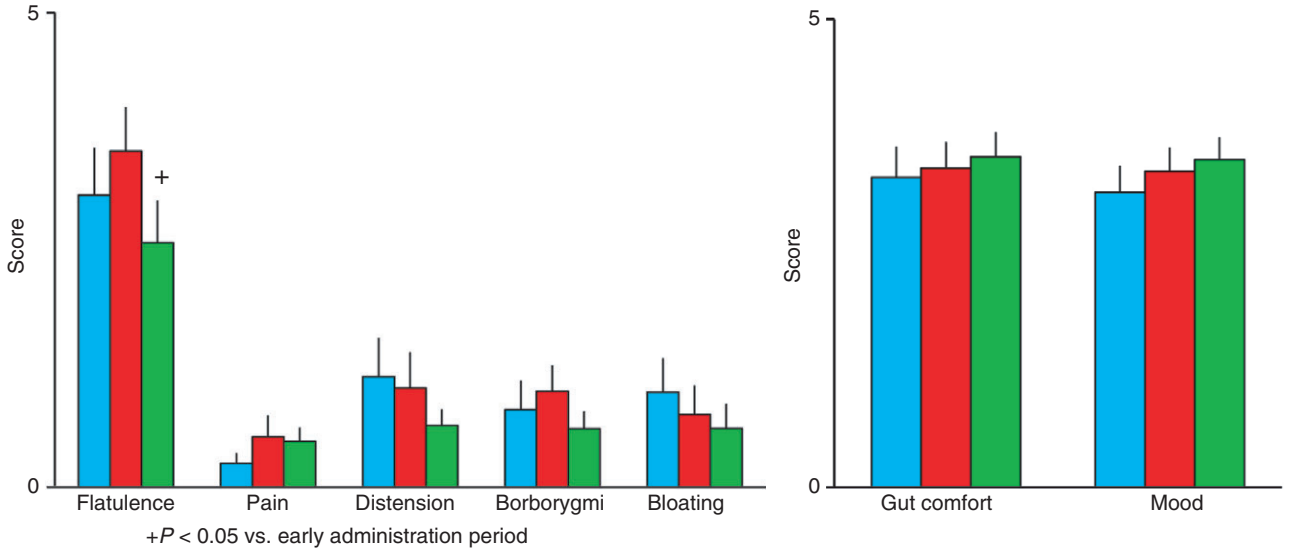


Figure 2 | Symptoms measured by daily questionnaires pre-administration (day −2 to 0, blue), in the early administration period (day 1–3, red), and in the late administration period (day 19–21, green) (n = 20). Data are average over each 3-day periods.

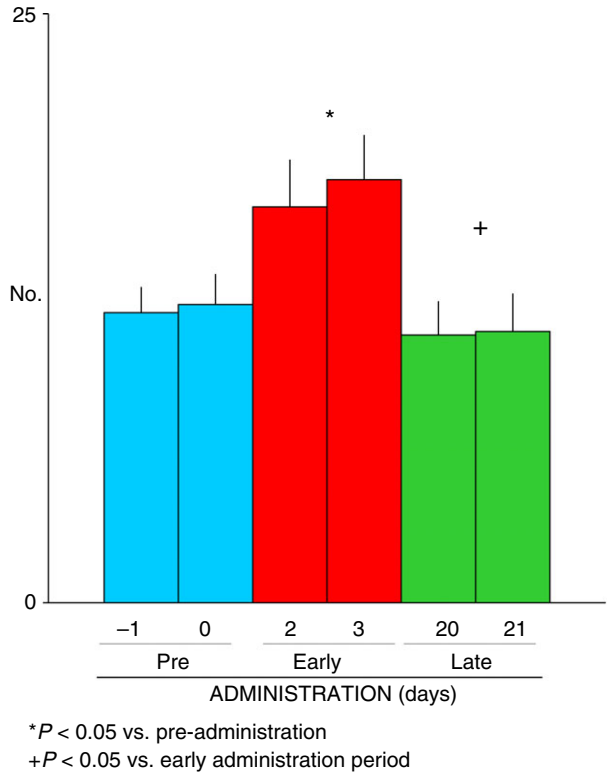


Figure 3 | Number of daytime anal gas evacuations during the last 2 day of the evaluation periods: pre-administration, in the early treatment period and the late administration period (n = 20).

Likewise, the volume of gas collected after the probe meal increased by 64 ± 16%; up to 236 ± 23 mL (P = 0.006 vs. pre-administration) (Figure 4), but this did not affect sensation scores measured during the gas-collection period after the probe meal (Figure 5).

Late administration period. After 3 weeks of HOST-G904 administration, on the standard diet the abdominal sensation were not significantly different than before administration (Figure 2). After 3 weeks of administration, the number of anal gas evacuations on the standard diet (Table 2) significantly decreased as compared to the early administration period (P = 0.001) and returned to the pre-administration level (11 ± 2 daytime evacuations; P = 0.351 vs. pre-administration) (Figure 3). The same adaptive effect was observed on the volume of gas evacuated after the probe meal (169 ± 23 mL; P = 0.002 vs. early administration; P = 0.733 vs. pre-administration) (Figure 4).

Microbial changes during the intervention

Cluster analysis of the microbial profiles in the 60 faecal samples (three time points per subject) was performed using the unweighted UniFrac principal coordinates analysis (PcoA) (Figure 6). Samples of the three time points clustered together in most subjects, indicating that intra-individual fluctuations of the microbiota during

the intervention were less distinctive than the inter-individual differences. Changes in composition during HOST-G904 administration followed different patterns in different individuals and no significant statistical differences in specific taxa were found when comparing day 0 vs. day 3 or day 21 samples in the overall group of 20 subjects. However, the relative abundance of bifidobacteria increased in 13 subjects with low levels at baseline, as defined by relative abundance below 0.5% of total bacteria. In these subjects (n = 13) abundance of bifidobacteria at day 3 (0.49 ± 0.23%) and at day 21 (0.28 ± 0.10%) was significantly higher than at day 0 (0.14 ± 0.06%; P = 0.042 and P = 0.031, respectively); values at day 3 and day 21 were not significantly different (P = 0.200). The remainder seven individuals with abundance above 0.5% at baseline showed no significant changes in bifidobacteria. There were no differences in gas volumes between subjects who increased bifidobacteria numbers during HOST-G904 consumption (from day 0 to day 21) and those with stable abundance.

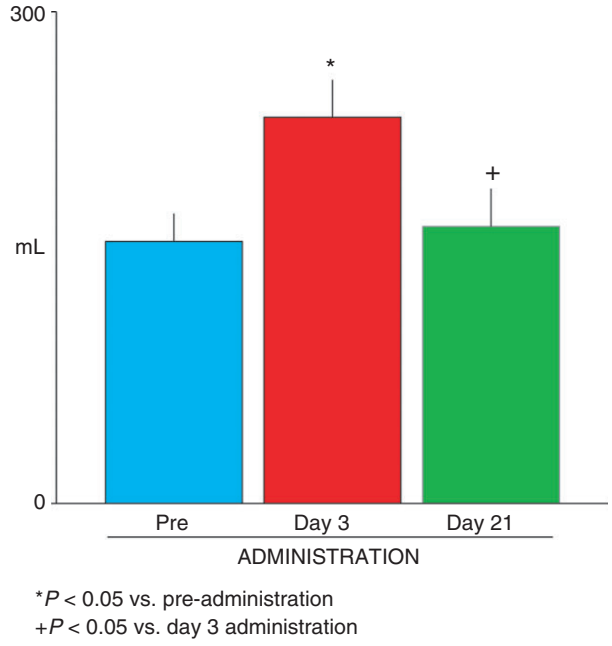


Figure 4 | Volume of gas evacuated in response to probe meal pre- administration, in the early treatment period (day 3), in the late administration period (day 21) (n = 20).

Volumes of gas recorded at day 21 correlated inversely with abundances of Lachnospiraceae (r = −0.52, P = 0.02), Clostridiaceae (r = −0.41, P = 0.07) and an unknown clostridiales species (r = −0.45, P = 0.04) in faecal samples at day 21. In addition, seven subjects harbouring methanogens (Methanobrevibacter) also produced low volumes of gas at day 21, although the correlation in the whole group (n = 20) was not significant due to the fact that in 13 subjects methanogens were negligible. Figure 7 shows the 3D display of abundances of Methanobrevibacter, Lachnospiraceae and Clostridiaceae, where the black solid dots represent the 5

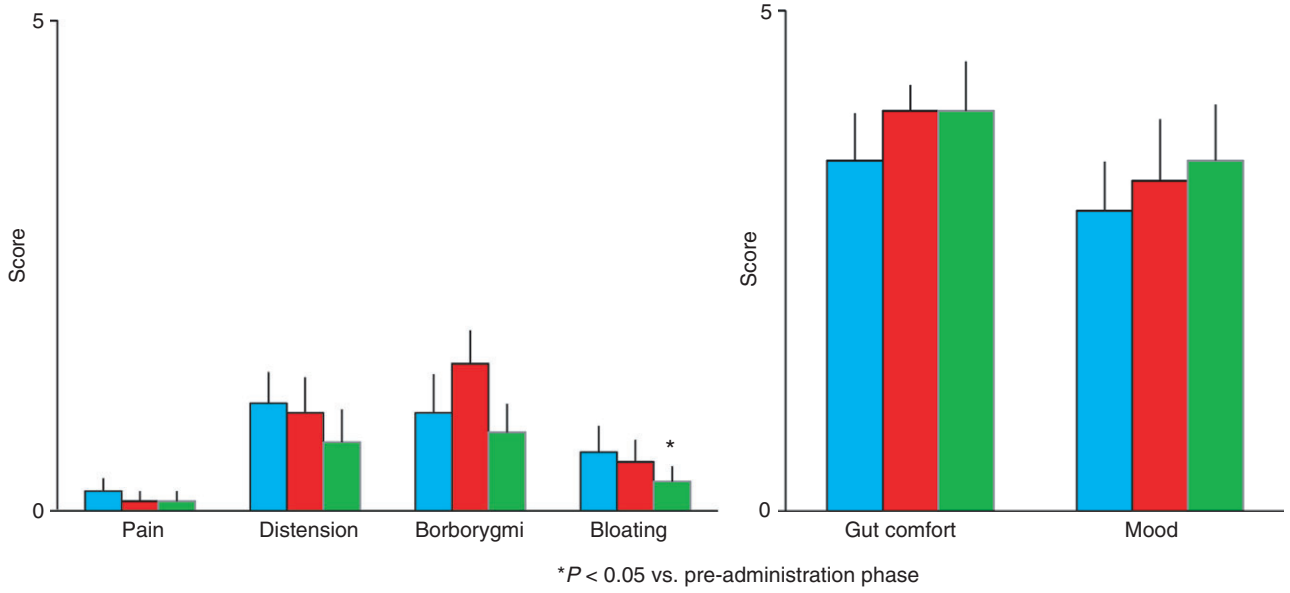


Figure 5 | Symptoms in response to probe meal pre- administration (blue), in the early administration period day 3 (red), in the late administration period day 21 (green) (n = 20).

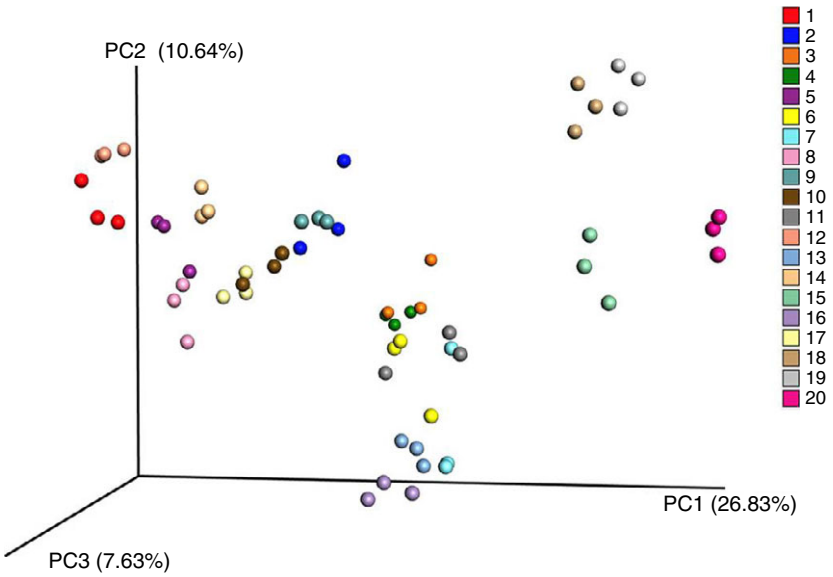


Figure 6 | Principal coordinates analysis of the microbial profiles in faecal samples from 20 subjects at 3 time points (day 1, 3 and 21). Samples from the same subject (same colour) clustered together in most cases, indicating that intra-individual fluctuations of the gut microbiota during the intervention were less distinctive than the inter-individual differences.

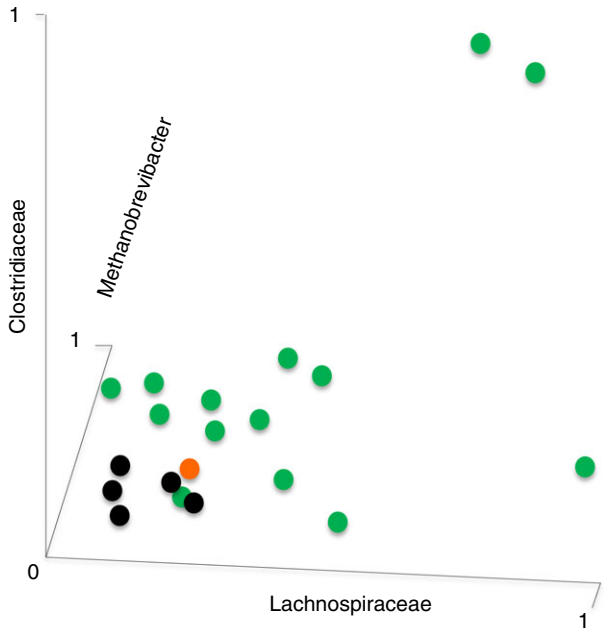


Figure 7 | A three-axis plot shows relative abundance of *Methanobrevibacter, Lachnospiraceae* and *Clostridiaceae* in faecal samples at day 21. The black solid dots are the samples from five subjects who produced more than 200 mL of gas at day 21. The remainder 15 subjects produced less than 200 mL of gas, and the red dot is a sample with high abundance of an unknown clostridiales species.

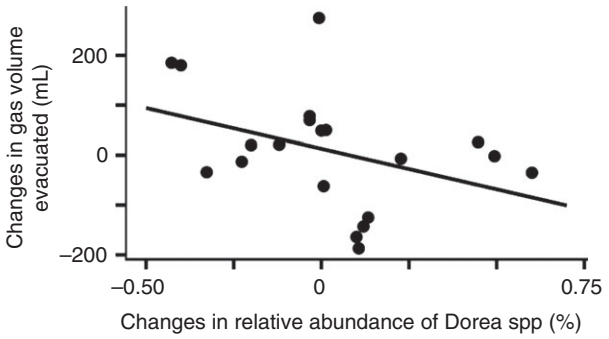
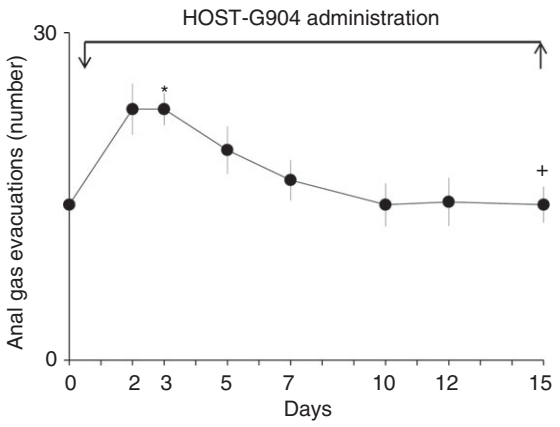


Figure 8 | Inverse correlation ($r = -0.48$; $P = 0.03$) between changes in gas volume from day 0 to 21, and changes in the abundance of *Dorea* spp. from day 0 to 21. Increases in *Dorea* spp. were associated to reduced gas production by the end of the prebiotic administration period.

subjects who produced more than 200 mL of gas at day 21. Thus, the five individuals with high gas production after 21 day prebiotic consumption had low abundance of the above-mentioned taxa; in contrast, in the remainder 15 subjects with low gas production at day 21 (<200 mL), gas production had decreased during the administration period, and 14 of them showed higher abundance of methanogens, Lachnospiraceae, Clostridiaceae or an unknown clostridiales species (Figure 6). Finally, Figure 8 shows changes relative to baseline in gas production and in abundance of the genus *Dorea* in faeces. An inverse correlation ($r = -0.48$, $P = 0.03$) suggests that increases in *Dorea* spp. were associated to reduced gas production by the end of the prebiotic administration period. Lachnospiraceae, Clostridiaceae and *Dorea* species ferment sugars and produce organic acids.



*P = 0.015 vs. day 0; + P = 1 vs. day 0 and P = 0.007 vs. day 3

Figure 9 | Control study. Time effect of HOST-G904 administration on number of daytime anal gas evacuations measured in the ancillary study (n = 6).

Ancillary study: external control group

With participants on their habitual diet, the number of anal gas evacuations significantly increased at the beginning of HOST-G904 administration and gradually declined back to the baseline level by 10 day administration (Figure 9).

DISCUSSION

Our data demonstrate the adaptation of gut microbiota to the intraluminal environment: increased availability of HOST-G904 led to a change in microbiota that had more efficient metabolic routes. High volumes of gas correlated with low methanogenic populations in some participants. This may be explained by increases in hydrogen, which is the usual substrate for gut-derived methane.[34, 35] Conversely, lower gas production was concomitant with high methanogenic populations, presumably as hydrogen was converted to methane.

The effect of HOST-G904 administration was tested with participants on a standardised diet, that is, a diet low in fermentable residues, as previously tested in our laboratory,[5] but complemented with fixed portions of a choice of foodstuffs containing equivalent amounts of fibre that were also previously used in a high-flatulogenic diet.[4] Under these conditions, the amount of daytime anal gas evacuations was in the expected range based on previous data from healthy subjects under various dietary regimes.[4, 5, 36] Similarly, the volume of gas evacuated per anus measured for 4 h after a low-residue probe meal fit the anticipated values derived from previous observations under various experimental conditions.[4, 5, 36] Interestingly, a relatively small supplement with a non-absorbable product (2.8 g/day HOST-G904) in addition to the dietary fibre intake, initially produced a marked effect on the microbiota metabolic activity, as reflected by gas production and as measured by the number of daytime anal gas evacuations and volume of gas evacuated after the probe meal. The effect of HOST-G904 on the microbiota metabolic activity was already present 24 h after the first administration without major changes observed 24 h later. The colonic transit time of inert residues averages 35 h in healthy subjects[37, 38]; conceivably, HOST-G904 was consumed at an earlier stage before reaching the distal colon.

The volume of intestinal gas produced depends in part on the amount of fermentable residues reaching the colon and the metabolic pathways used by the microbiota to consume them.[39] Hence, on the same diet, the amount of gas produced differs among individuals as a function of their microbiota profile. A portion of the gas produced by fermentation is consumed by other pools of microorganisms in the formation of less oxidised products, a portion is absorbed into the blood and cleared by breathing, and the rest is evacuated per anus.[39] At the first exposure to HOST-G904, the increase in gas production was conceivably related to the availability of substrates. A decrease in anal gas evacuation after adaptation, that is, a decrease in net gas production, could be related to the proliferation of microorganisms using non-fermentative pathways to metabolise the substrates with less gas production and/or to the up-regulation of the gas-consuming activity. Indeed, reduced gas production was related to the proliferation of methanogens that use H_2 to reduce CO_2 to CH_4, reducing the volume of gas by 1:5.[39] Conversely, individuals with low counts of methanogens and of some specific organic acid producers exhibited a poor adaptation at the end of the administration period.

To ensure similar testing conditions within and between individuals, participants were put on standardised diet during the 3-day evaluation periods before, at the beginning and at the end of prebiotic administration. We wish to acknowledge that diet standardisation may have different effects depending on individuals′ habitual diet with potential increase or decrease in fibre intake, and this might interfere with the effect of the prebiotic. To account for this potential limitation, the prebiotic was also tested in an external control group of subjects on their habitual diet and the same response, in terms or number of anal gas evacuations, was observed. We acknowledge the inherent limitations and potential bias

of external controlled trials, and that in the control group the volume of gas evacuated and microbiota were not measured.

It is interesting that a relatively small amount of HOST-G904 relative to the daily dietary fibre intake had a remarkable effect, initially on microbiota metabolic activity and subsequently inducing adaptation. Conceivably, not all fermentable residues have the same capability; the power to induce adaptation might be a crude indicator of prebiotic activity. We based the test dose on previous studies[9]; a higher dose might compromise selectivity of fermentation, which is a requirement for prebiotic effects. The dose of HOST-G904 that was used activated microbiota metabolism and increased the number of anal gas evacuation without inducing abdominal symptoms in healthy subjects. We cannot ascertain how this dose would be tolerated by patients with functional gut disorders, but conceivably, potential symptoms at first exposure would subside with adaptation. The ancillary study showed that the number of daily anal gas evacuations started to decrease after 5 day administration and by 10 day returned to pre-administration level; it remains to be determined whether symptom adaptation in patients follows the same time pattern.

HOST-G904 has been shown to improve symptoms in patients,[9] which was attributed to beneficial changes in gut microbiota. Our study in healthy subjects has potential clinical implications. Indeed, our data would support advising patients to allow for a period of adaptation before prebiotic effects become apparent.

AUTHORSHIP

Guarantor of the article: Fernando Azpiroz.

Author contributions: MM: study management, conduction of experiments, and data analysis; CM. microbiota analysis, manuscript revision; AA: supervision of studies; DC: microbiota analysis; MP: microbiota analysis; EV: microbiota analysis; JV: study design, interpretation of results, manuscript revision; GT: study design, interpretation of results, manuscript revision; GG: study design, interpretation of results, manuscript revision; FG: study design, interpretation of results, manuscript revision; FA: study design, data interpretation, and manuscript preparation.

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