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Randomized Control Trials

Galacto-oligosaccharides supplementation in prefrail older and healthy adults increased faecal bifidobacteria, but did not impact immune function and oxidative stress

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SUMMARY

Background & aims: Ageing is associated with an increased risk of frailty, intestinal microbiota perturbations, immunosenescence and oxidative stress. Prebiotics such as galacto-oligosaccharides (GOS) may ameliorate these ageing-related alterations. We aimed to compare the faecal microbiota composition, metabolite production, immune and oxidative stress markers in prefrail elderly and younger adults, and investigate the effects of GOS supplementation in both groups.
Methods: In a randomised controlled cross-over study, 20 prefrail elderly and 24 healthy adults received 21.6 g/day Biotis™ GOS (containing 15.0 g/day GOS) or placebo. Faecal 16S rRNA gene-based microbiota and short-chain fatty acids were analysed at 0, 1 and 4 weeks of intervention.Volatile organic compounds were analysed in breath, and stimulated cytokine production, CRP, malondialdehyde, trolox equivalent antioxidant capacity (TEAC) and uric acid (UA) in blood at 0 and 4 weeks.
Results: Principle coordinate analysis showed differences in microbial composition between elderly and adults ($P \leq 0.05$), with elderly having lower bifidobacteria ($P \leq 0.033$) at baseline. In both groups, GOS affected microbiota composition ($P \leq 0.05$), accompanied by increases in bifidobacteria ($P < 0.001$) and decreased microbial diversity ($P \leq 0.023$). Faecal and breath metabolites, immune and oxidative stress markers neither differed between groups ($P \geq 0.125$) nor were affected by GOS ($P \geq 0.236$). TEAC values corrected for UA were higher in elderly versus adults ($P < 0.001$), but not different between interventions ($P \geq 0.455$).
Conclusions: Elderly showed lower faecal bifidobacterial (relative) abundance than adults, which increased after GOS intake in both groups. Faecal and breath metabolites, parameters of immune function and oxidative stress were not different at baseline, and not impacted by GOS supplementation.
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1. Introduction

In line with a continuously rising life expectancy, the ageing population is increasing worldwide. Ageing is associated with a decline in general physiological functioning, including immunosenescence, contributing to frailty [1]. By applying the Fried criteria to determine physical frailty, a recent study showed prevalence rates of 50.5% and 16.0% for prefrail and frail status, respectively, in a

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Abbreviations

ALT	alanine transaminase
ASCA	ANOVA-simultaneous component analysis
ASVs	amplicon sequence variants
UROC	under the curve of receiver operating characteristic
CRP	c-reactive protein
DCA	detrended correspondence analysis
FDR	false discovery rate
FISH	fluorescent in situ hybridisation
GCP	good clinical practice
GI	gastrointestinal
GGT	gamma-glutamyl transpeptidase
GOS	galacto-oligosaccharides
GSRS	gastrointestinal symptom rating scale
HPLC	high performance liquid chromatography
IFN	interferon
IL	interleukin
IQR	interquartile range
LPS	lipopolysaccharides
MDA	malondialdehyde
PBMC	peripheral blood mononuclear cells
PCoA	principle coordinate analysis
PCR	polymerase chain reaction
PD	phylogenetic diversity
PERMANOVA	permutational multivariate analysis of variance
PHA	phytohemagglutinin-M
qPCR	quantitative PCR
RDA	redundancy analysis
rRNA	ribosome RNA
SCFAs	short-chain fatty acids
SD	standard deviation
TEAC	trolox equivalent antioxidant capacity
TNF	tumor necrosis factor
UA	uric acid
uRF	unsupervised Random Forest
VOCs	volatile organic compounds

European population of community-dwelling elderly (aged 75–84 years) [2]. Frailty has been associated with impaired quality of life, increased risk of comorbidity and increased healthcare costs [3].

The intestinal microbiota comprises a complex community of microbes dominated by anaerobic bacteria, and plays a key role in intestinal immunity and defense capacity [4], either directly by microbe-cell interactions or indirectly via bacterial metabolites. Lower microbial diversity, richness and alterations in relative abundance of several bacterial groups have been found in elderly, being most pronounced in frail elderly and in association with lifestyle risk factors [5]. The mechanisms through which these changes in the intestinal microbiota are related to age-related immunosenescence remain unclear up to now [6]. Immunosenescence refers to age-related alterations in immune function, and is characterised by *e.g.* dysfunctional immune cells such as monocytes and T-cells, and a pro-inflammatory cytokine profile [7]. Moreover, immunosenescence is associated with oxidative stress [8], a condition in which the balance between pro- and antioxidants is disrupted leading to an oxidizing state [8,9]. Both immunosenscence and higher oxidative stress levels are considered risk factors for age-related morbidities such as infections [8], as well as for cardiovascular [10] and neurodegenerative [11] disorders.

Since frailty is not a unidirectional process, early identification (*i.e.* prefrail status) and targeted interventions may help to improve health status and thereby decrease comorbidity and healthcare costs in elderly. One such approach could be via the intake of prebiotics such as galacto-oligosaccharides (GOS). GOS are non-digestible oligosaccharides, which have been classified as prebiotic because of their impact on the intestinal microbiota and host health. Increased production of short-chain fatty acids (SCFAs) after GOS intake may contribute to anti-inflammatory and antioxidant effects. Vulevic et al. [12] showed that administration of 5.5 g/day GOS for five weeks beneficially altered the faecal microbiota and some markers of immune function in healthy elderly. However, data on the effects of GOS in prefrail elderly are lacking.

The aims of the current study were twofold: 1) to compare prefrail elderly and healthy younger adults with respect to faecal microbiota composition, metabolites in faeces and exhaled breath, immune markers and oxidative stress markers, and 2) to study the impact of four weeks GOS supplementation in both groups. We hypothesised that faecal microbiota composition, metabolites in faeces and exhaled breath, and parameters of immune function and systemic oxidative stress show perturbations in prefrail elderly when compared with healthy adults, and that these markers can be beneficially altered by four weeks of GOS supplementation.

2. Materials and methods

The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center+ and registered in the US National Library of Medicine (http://www.clinicaltrials.gov, NCT03077529). The study was performed at the Maastricht University Medical Center + between March 2017 and September 2018, according to the Guidelines of the Declaration of Helsinki (latest amendment of 2013, Fortaleza, Brazil), Dutch Regulations on Medical Research involving Human Subjects and Good Clinical Practice (GCP) guidelines. All participants gave written informed consent before participation.

2.1. Subjects

The number of subjects in our study was determined based on a previous study investigating the effects of GOS on different bacterial groups in faeces of healthy elderly volunteers using fluorescent in situ hybridisation (FISH). in which the estimated effect after five weeks GOS intervention was an increase of 0.59 ± 0.44 log 10 cells/g faeces for bifidobacteria and a decrease of 0.018 ± 0.022 log10 cells/g faeces for *Bacteroides* spp [12]. Using these numbers with the power (1-β) set at 0.8, and the significance level (α) at 0.025 the sample size per age group equaled 8 and 17 subjects, respectively. This number is in line with a study described by O'Keefe et al. [13] in which it was shown that 20 subjects per group were sufficient to robustly identify differences in microbiota composition between low and high fibre intake. Taking all above into account, 20 subjects were included per age group, which should be sufficient to pick up changes in microbiota composition, as well as in specific genera.

Men and women without gastrointestinal (GI) complaints, Body Mass Index (BMI) 20–30 kg/m^2 and being weight stable were recruited in two age groups: elderly 70–85 years of age and adults 25–50 years of age. The Fried criteria to determine physical frailty [14] were used to classify and include prefrail elderly (score 1 or 2) and robust adults (score 0). Other inclusion criteria were

hemoglobin, C-reactive protein (CRP), creatinine, alanine transaminase (ALT) and gamma-glutamyl transpeptidase (GGT) within the normal range of reference values, or being slightly outside the normal range but not at risk of severe comorbidities (as determined by a medical doctor). Key exclusion criteria were history of any chronic disorder or major surgery, which potentially limited participation, completion of the study or interfered with the study outcomes; self-reported human immunodeficiency virus or lactose intolerance; use of antibiotics 90 days before the start of the study, average alcohol consumption of >14 alcoholic units per week, pregnancy, lactation, institutionalization (*e.g.* hospital or nursing home), use of probiotics, prebiotics or laxatives 14 days before the start of the study, and history of side effects towards prebiotic supplements. As medication use and comorbidities are more frequent in elderly they were not general exclusion criteria, but considered to reflect (prefrail) ageing. Accordingly, anticoagulation, antihypertensive and statins were allowed, but had to be on stable dosing at least 14 days before and during the study. Additionally, two elderly were using a stable dose of proton pump inhibitors before and during the study. Antibiotics, anti-inflammatory/immune suppressive drugs and spasmolytics were not allowed.

2.2. Study design

This study was designed as a randomised, double-blind, placebo-controlled, cross-over study with a 4–6 weeks wash-out period between the intervention periods (Supplementary Fig. 1). Per age group (*i.e.* elderly and adults), randomisation was performed with concealed block sizes of four, to assign participants to the placebo or the GOS intervention period. An independent person generated two randomisation lists of random allocations using a computerised program (http://www.randomization.com), which was stratified for gender. Both study participants and investigators were blinded to the interventions until analyses were completed. During the GOS intervention period of four weeks, participants received 21.6 g/day of Biotis™ GOS Powder (FrieslandCampina Ingredients, Amersfoort, the Netherlands), containing 15.0 g/day of pure GOS. The remaining component in the BiotisTM GOS Powder are lactose (23%), monosaccharides (glucose and galactose; 5%) and moisture (3%). In the placebo intervention period, 21.6 g/day of maltodextrin (GLUCIDEX® IT 12, Roquette Frères, Lestrem, France), which is completely absorbed in the small intestine, was supplemented for four weeks. We assume the remaining components (*i.e.* lactose, monosaccharides and moisture) in the BiotisTM GOS Powder are fully absorbed in the small intestine, just like the maltodextrin. Both GOS and placebo were provided as white powdered supplements with similar appearance (*i.e.* color, taste and odor), and packed in closed sachets of a single dose of 7.2 g. Participants were asked to ingest the content of three sachets daily (before breakfast, lunch and dinner), by transferring the powder in a glass, mixing with approximately 200 ml tap water and consuming the complete drink. Time of consumption had to be recorded, and empty sachets were returned to assess compliance. At baseline, after one week and after four weeks GOS and placebo intake, faecal samples were collected and three-day dietary records and stool characteristics scores were completed at home (Supplementary Fig. 1). Faecal samples were stored at −20 °C until arrival at the study site, and subsequently stored at −80 °C for later analyses. At baseline and after four weeks GOS and placebo supplementation, venous blood and exhaled air samples were collected after an overnight fast, and stored at −80 °C and room temperature, respectively, until further use. The GI symptom rating scale (GSRS) was completed at baseline and at weekly intervals. Supplement intake continued until all samples were collected and questionnaires completed.

2.3. Microbiota composition

Total DNA was isolated from faeces and subsequently purified as described previously [15]. Microbiota composition was determined by sequencing of barcoded 16S ribosomal RNA (rRNA) gene amplicons using Illumina Hiseq 2500 (2 × 150 bp). The V4 region of prokaryotic 16S rRNA genes was amplified in triplicate using 515F [16]-806R [17] primers and purified DNA as template. Polymerase chain reaction (PCR) was performed as described previously [15], with annealing temperature of 50 °C. An equimolar mix of purified PCR products was sent for sequencing (GATC-Biotech, Konstanz, Germany). Raw sequence data was processed using NG-Tax default settings [18,19]. Briefly, generated paired-end libraries were demultiplexed with pre-defined and matching barcodes, resulting in 248794 reads on average per sample. Amplicon sequence variants (ASVs) were picked as follows: 1) ordering all sequences by abundance per sample, 2) reads were considered valid when its cumulative abundance was smaller than user defined threshold (in current study 0.1%). Thereafter, the taxonomy assignment to each ASV was conducted using the USEARCH algorithm [20] based on the Silva database (v128) [21]. The sequencing data has been deposited into European Nucleotide Archive with accession number PRJEB32547.

2.4. qPCR analysis

Total DNA was also used for quantitative PCR (qPCR) using a CFX384 Touch ™ Real-Time PCR Detection System (Bio-Rad, California, USA). The reaction mixture was composed of 6.25 μl iQ ™ SYBR ® Green Supermix, 0.25 μl forward primer (10 μM), 0.25 μl reverse primer (10 μM), 3.25ul nuclease free water and 2.5 μl DNA template. Primers were used that targeted either total bacteria [22] or *Bifidobacterium* spp [23]. The program for amplification of total bacteria was initiated at 95 °C for 3 min, followed by 40 cycles of denaturing at 95 °C for 15 s, annealing at 52 °C for 30 s and elongation at 72 °C for 30 s. The program for total bifidobacteria was: 94 °C for 5 min, followed by 40 cycles of 94 °C for 20 s, 55 °C for 50 s, 72 °C for 50 s. Both programs were ended with a melt-curve analysis from 60 °C to 95 °C with 0.5 °C per step. Reactions for the quantification of total bacteria and total bifidobacteria were performed in triplicates. Data was analysed using the CFX manager ™ (Bio-Rad).

2.5. Faecal metabolites

Faecal organic acids resulting from microbial fermentation were measured using high performance liquid chromatography (HPLC), equipped with a SUGAR SH1011column (Shodex, Japan). The column was operated at 45 °C, with a flow rate of 0.8 ml/min, using 0.1N H_2SO_4 as eluent. The compounds were detected by an RID-20A (Shimadzu, Kyoto, Japan) refractive index detector at a temperature of 40 °C. One gram faeces was suspended in 4.0 ml Milli-Q water, mixed and centrifuged at 4 °C 2000×*g* for 20 min. Four hundred μl of supernatant was mixed with 600 μl of 10 mM DMSO in 0.1N H_2SO_4. Ten μl of samples was injected and subjected to analysis. The chromatograms were depicted and analysed with Chromeleon™ Chromatography Data System (CDS) Software (ThermoFisher Scientific, Massachusetts, USA). Organic acids were expressed per gram dry matter to correct for stool consistency. Dry matter content

was determined by vacuum drying of 500 mg faeces for 5 h at 60 °C (Concentrator plus, Eppendorf, Hamburg, Germany).

2.6. Volatile organic compounds

Metabolites were also analysed in exhaled breath. Therefore each participant delivered exhaled air samples by breathing into a 3L Tedlar bag (SKC Limited, Dorset, UK). The content of the bag was transferred within 1 h to carbon-filled stainless steel absorption tubes (Markes International, Llantrisant, UK) using a vacuum pomp (VWR international, Radnor, PA, USA). Volatile organic compounds (VOCs) captured into stainless tubes were measured using thermal desorption gas chromatography coupeled to time-of-flight mass spectrometry (GC-*tof*-MS, (Markes International, Llantrisant, UK) as described previously [15,24]. The resulting breath-o-grams were denoised, baseline corrected, aligned, normalised by probabilistic quotient normalisation and scaled for further analyses [25].

2.7. Immune parameters

Blood was collected in BD Vacutainer® sodium heparin and serum tubes (BD Biosciences, San Jose, CA, USA). Sodium heparin blood was used within 3 h for whole blood stimulations to determine *ex vivo* cytokine production. RPMI 1640 medium with HEPES and glutamax (Thermo Fischer Scientific, Waltham, MA, USA), supplemented with 10% heat inactivated Fetal Bovine Serum (Sigma–Aldrich, St. Louis, MO, USA) and 1% Penicillin/Streptomycin (Gibco™, Thermo Fischer Scientific, Waltham, MA, USA) was used as culture medium. Whole blood was mixed with medium in 1:5 ratio, and incubated with 10 μg/ml Phytohemagglutinin-M (PHA) (L8902, Sigma–Aldrich, St. Louis, MO, USA) to stimulate T-cells, and 10 μg/ml E. *coli* 055:B5 Lipopolysaccharides (LPS) (L4524, Sigma–Aldrich, St. Louis, MO, USA) to stimulate monocytes. After 24 h incubation at 37 °C and 5% CO_2, samples were centrifuged (8 min, 283×*g*) and plasma aliquots were stored at −80 °C for further analyses. Plasma samples were thawed and kept at 4 °C until incubation with Interleukin (IL)-1β, IL-6, IL-8, IL-10, Interferon (IFN)-γ and Tumor Necrosis Factor (TNF)-α antibodies (Catalogue numbers 558279, 558276, 558277, 558274, 558269 and 560112, respectively, BD Biosciences, San Jose, CA, USA). The Cytometric Bead Array (BD Biosciences, San Jose, CA, USA) was performed according to manufacturer's instructions and samples were measured using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) and analysed with FACSDiva ™ Software (BD Biosciences, San Jose, CA, USA). Serum CRP concentrations were determined by immunoturbidimetric assay using Cobas 6000 analyzer (Roche, Mannheim, Germany).

2.8. Parameters of systemic oxidative stress

Lithium heparin blood (BD Vacutainer®, BD Biosciences, San Jose, CA, USA) was collected for malondialdehyde (MDA), trolox equivalent antioxidant capacity (TEAC) and uric acid (UA) analyses. MDA plasma concentrations were quantified after derivatisation with thiobarbituric acid into a fluorescent chromogen [26]. The chromogen was extracted from the samples with butanol after centrifugation at 30,000×*g* for 5 min. Fluorescence of the butanol phase was measured in black bottom 96-wells plates by using an excitation wavelength of $\lambda_{ex} = 530$ nm and an emission wavelength of $\lambda_{em} = 560$ nm. Plasma antioxidant capacity was quantified as TEAC values, according to Fischer et al. [27] and corrected for inter-individual differences in plasma UA concentrations [28] quantified by HPLC [29].

2.9. Dietary intake

Participants were asked to complete dietary records on three consecutive days before each test day. Before the start of the first intervention period, participants were instructed how to record their food, beverage and dietary supplement intake based on standard household units. Energy and nutrient intake were analysed using the online dietary assessment tool of The Netherlands Nutrition Centre (www.voedingcentrum.nl). As polyphenols are a major class of dietary antioxidants in addition to some vitamins and minerals, their intake was also calculated using PhenolExplorer 3.6, a comprehensive database containing polyphenol content values of over 400 food items [30,31]. Recruited subjects were required to maintain their habitual diet throughout the study.

2.10. Gastrointestinal tolerance and stool characteristics

The GSRS was used to monitor GI tolerance. It consists of 15 items using a seven-point Likert-type scale (where 1 represents absence of troublesome symptoms and 7 represents very troublesome symptoms) and calculated into five subscales including reflux, abdominal pain, indigestion, diarrhoea, and constipation [32]. Stool characteristics (*i.e.* stool frequency and stool consistency) were scored on three consecutive days using the Bristol Stool Scale. Stool consistency is an ordinal scale score ranging from hard (type 1) to soft (type 7). Frequencies of hard stools and loose stools were calculated and analysed as described previously [33].

2.11. Statistical analyses

Per parameter, we compared elderly and adults, and subsequently identified effects of GOS versus placebo interventions in both age groups. For this, normality of the data was tested by histograms and summarised accordingly using the median and interquartile range (IQR; 25–75th IQR) or means ± standard deviation (SD) for numerical variables, and by percentages for categorical variables. Subsequently, independent-sample t-tests and Mann–Whitney U tests were performed for numerical variables, and Chi-square tests for categorical variables to test for differences between elderly and adults.

The 16S rRNA gene sequence read counts were normalised to relative abundance and the microbial alpha-diversity indices (Faith's phylogenetic diversity (PD) and Inverse Simpson) were calculated based on ASVs as implemented in Phyloseq and vegan packages [34,35]. Homogenicity of the dataset was evaluated by detrended correspondence analysis (DCA) and subsequently using linear or unimodal constrained ordination for multivariate analysis. Redundancy analysis (RDA) was used to assess the contribution of environmental variables (*e.g.* BMI, age, alcohol, medication *etc.*) to the observed microbiota variation. In addition, since average difference in age coincided with average difference in BMI, a regression analyses was perfomed to determine whether difference in BMI could explain the difference in the absolute (qPCR) and relative (amplicon seuqence reads) abundance of *Bifidobacterium*. Principle coordinate analysis based on weighted UniFrac, unweighted UniFrac, Bray–Curtis and Jaccard distances was used to evaluate variation in microbiota composition at ASVs level. Significance of

observed differences between groups was tested by permutational multivariate analysis of variance (PERMANOVA). Intervention effects on single bacterial taxa and microbiota diversity indices were evaluated using a linear mixed model as implemented in the *nlme* package [36], with variance components (random intercept) and correction for baseline values. All statistical analyses of the microbiota data were conducted in R (R-3.5.0).

Exhaled breath data were analysed with ANOVA-simultaneous component analysis (ASCA) [37] and unsupervised Random Forest (uRF) [38]. The ASCA model was used to investigate the effect of the treatment and uRF for visualisation of the data. Data were log transformed to account for data skewed distribution and pareto-scaled to ensure equal contribution of each volatile metabolite in breath in the ASCA analysis. In case of uRF analysis data were only log transformed. The statistical analysis was carried out separately for each age group. The differences in VOC profiles at baseline between elderly and adults were explored using a supervised verion of RF analysis. The training set was used to find the discriminatory VOCs and to build the classification model. The performance of the RF classification model was expressed by the area under the curve of receiver operating characteristic (AUROC) for the validation set. Statistical analyses of VOCs were performed using Matlab 2019a (The MathWorks, Natick, 2018).

Both intention to treat and per protocol analyses were performed. Within age groups, differences between intervention periods were assessed by variance components (random intercept) linear mixed model analyses with intervention group, time, intervention period, 'intervention group × time', 'intervention group × intervention period', and 'time × intervention period' as fixed factors, and correction for baseline values. For significant intervention effects, differences between age groups were assessed by addition of the fixed factor 'intervention × time × age group'. All statistical analyses were performed for adults and elderly separately using IBM SPSS Statistics for Windows (version 25.0, IBM Corporation, Armonk, NY, USA). *P*-values ≤ 0.05 (two-sided) were considered to indicate statistical significance. *P*-values were corrected for multiple testing by the false discovery rate (FDR) of Benjamini–Hochberg per cluster of parameters (*i.e.* bacterial taxa, metabolites, immune, oxidative stress and dietary intake) per age group. GI symptoms were corrected by FDR for multiple time points.

3. Results

3.1. Study subjects, GI tolerance and dietary intake

After evaluating 66 elderly and 33 adults for eligibility, 20 elderly (all with Fried frailty score 1) and 24 adults (Fried frailty score 0) were randomised in the study. Three adults dropped out and one adult was non-compliant to the intervention (Supplementary Fig. 2). Elderly and adults returned on average 97.6% and 95.0% of the empty sachets, respectively.

As per protocol analyses led to the same conclusions, only the results of the intention to treat analyses are shown. Concerning baseline characteristics, besides age, also medication use and BMI were significantly different between age groups (Table 1).

GI tolerance towards the intervention products was assessed weekly by use of the GSRS questionnaire. After FDR correction for multiple testing, GI symptom scores were not significantly different between GOS and placebo intervention in elderly nor in adults (all $P \geq 0.058$) (Supplementary Fig. 3). In addition, average stool frequency as well as average frequencies of hard stools and loose stools were not significantly different between GOS and placebo supplementation in elderly and adults after FDR correction for multiple testing (all $P \geq 0.170$).

Table 1
Baseline characteristics of the elderly (n = 20) and adults (n = 24).

	Elderly (n = 20)	Adults (n = 24)	*P*-value
Age (yrs, mean ± SD)	74.3 ± 3.7	38.2 ± 7.8	<0.001
Sex (% female)	45.0	66.7[a]	0.149
BMI (kg/m^2, mean ± SD)	26.4 ± 3.0	23.1 ± 2.6	<0.001
Smoking (%)	10.0	12.5	0.795
Habitual alcohol consumption (%)			
<1 unit/wk	25.0	33.3	0.251
1–7 units/wk	55.0	62.5	
8–14 units/wk	20.0	4.2	
Medication use (%) [b]			
No	60.0	95.8	0.003
Anticoagulation	25.0	4.2	0.045
Antihypertensives	25.0	0	0.009
Statins	15.0	4.2	0.213
Blood parameters (mean ± SD)[c]			
Hemoglobin (mmol/L)	8.8 ± 0.8	8.8 ± 0.7	0.984
CRP (mg/L)	1.1 ± 1.3	1.5 ± 3.1	0.592
Creatinin (μmol/L)	83.3 ± 17.6	74.8 ± 12.4	0.068
ALT (U/L)	22.5 ± 10.1	23.3 ± 9.2	0.787
GGT (U/L)	22.5 ± 11.1	20.8 ± 11.9	0.629

ALT: alanine transaminase, BMI: body mass index, CRP: C-reactive protein, GGT: gamma-glutamyl transpeptidase.
[a] Drop-outs and non-compliant subject were all female, and replaced by females.
[b] Most commonly used medication.
[c] Taking into account gender specific reference values, five adults and seven elderly showed a limited increase or decrease in one of the blood parameters not associated with clear comorbidity. Age, BMI and blood parameters were compared between intervention groups with the use of an independent samples *t*-test. Sex, smoking, habitual alcohol consumption and medication use were compared between intervention groups with the use of a Pearson's Chi-square test.

Energy, macronutrient, micronutrient as well as polyphenol intake levels did not differ significantly between elderly and adults at baseline nor after GOS as compared to placebo supplementation (Supplementary Table 1).

3.2. Faecal microbiota composition

Principle coordinate analysis (PCoA) based on Bray–Curtis (considering relative abundance of ASVs, Supplementary Fig. 4A) and weighted UniFrac (considering relative abundance of ASVs and their position in the phylogenetic tree) revealed significant differences between the microbiota of adults and that of elderly (Fig. 1A), with 3.49% and 4.53% of variation explained by age groups at baseline, *i.e.* at the start of the first intervention period. However, PCoA based on Jaccard (only based on presence or absence) and unweighted UniFrac (based on presence or absence, and their position in the phylogenetic tree) distances showed no significant differences (Supplementary Fig. 4B and C), indicating the differences in microbiota profile are mainly driven by differences in relative abundance rather than presence/absence of bacterial taxa. We investigated the differences between elderly and adults, and the effects of GOS on genus level (Table 2). Only the relative abundance of *Bifidobacterium* was significantly lower in elderly compared with adults ($P = 0.033$, Table 2), suggesting that this group mainly contributed to the significant difference between the microbiota of adults and that of elderly. This was confirmed by qPCR ($P = 0.025$, Fig. 1C). Moreover, regression analyses demonstrated no significant contribution of BMI ($P > 0.05$) to the absolute (qPCR) and relative (amplicon seuqence reads) abundance of *Bifidobacterium*. RDA demonstrated no significant ($P > 0.05$) contribution of medication to the baseline microbiota variation. The relative abundance of all the other genera as well as microbial richness and diversity (both $P = 0.942$, Supplementary Fig. 4) were not significantly different between elderly and adults.

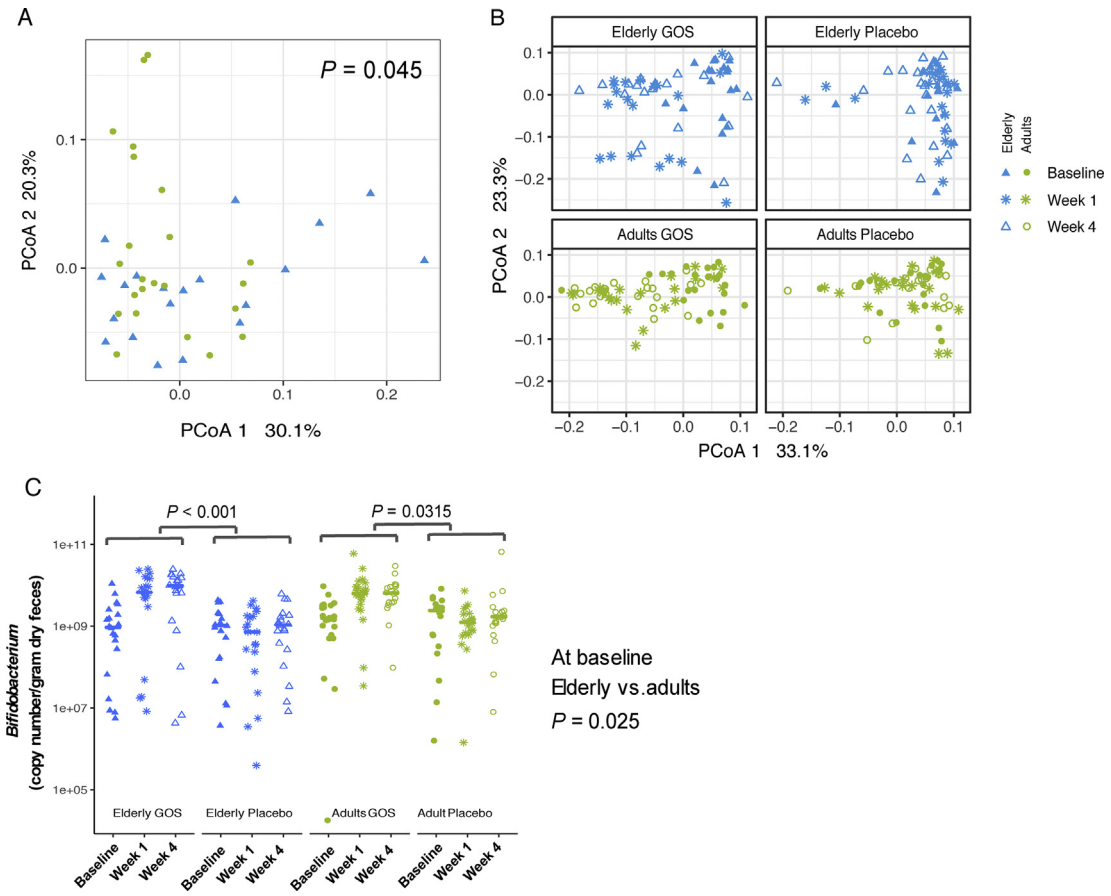

Fig. 1. Faecal microbiota at baseline (A), and comparing baseline microbiota to that after 1 week and 4 weeks GOS and placebo intervention (B) in 20 elderly and 23 adults based on 16S rRNA gene sequences. PCoA plots are based on weighted UniFrac distance matrice. PERMANOVA was used to compare the microbiota of elderly and adults in the PCoA plots at baseline, as well as for intervention effects between time points per group of subjects (*e.g.* Elderly baseline GOS vs Elderly Week 1 GOS). (C) *Bifidobacterium* (copy number/g dry faeces) based on qPCR, pre and post GOS and placebo intervention periods in 20 elderly and 23 adults. Sample sizes vary due to drop-outs and technical reasons. Values are presented in scatter plots with median line. Elderly vs. adults at baseline of the first intervention period were compared by a Mann–Whitney U test. Within age groups, interventions were compared with variance components (random intercept) linear mixed models and correction for baseline values. PCoA; Principle coordinate analysis.

PCoA based on weighted UniFrac (Fig. 1B) indicated that microbiota composition was significantly different after one and four weeks of GOS supplementation, both in adults (both $P = 0.003$) as well as elderly ($P = 0.003$ and $P = 0.010$, respectively), when compared to the corresponding data from subjects consuming the placebo supplementation. Similarly for Bray–Curtis distance based analysis (Supplementary Fig. 4D), microbiota profiles differed significantly after one and four weeks of GOS supplementation both in adults (both $P = 0.003$) and elderly ($P = 0.003$ and $P = 0.005$, respectively). However, no significant differences were observed based on unweighted UniFrac and Jaccard distance (Supplementary Fig. 4E and F; ($P > 0.05$)). The relative abundance of *Bifidobacterium* increased significantly after one and four weeks GOS supplementation in elderly and adults (both $P < 0.001$, Table 2), which was confirmed by qPCR for elderly ($P < 0.001$) and adults ($P = 0.0315$) (Fig. 1C). Furthermore, the relative abundance of bifidobacteria (illustrating intervention effects) in the group of subjects receiving GOS in the first intervention period, dropped back to baseline levels after four weeks of wash-out, indicating the absence of carry-over effects after GOS supplementation (Supplementary Fig. 5). The relative abundance of all the other genera was not significantly different between GOS versus placebo neither in elderly, nor in adults ($P > 0.05$). Microbial diversity (InvSimpson) decreased significantly after four weeks GOS supplementation in both adults ($P = 0.011$) and elderly ($P = 0.023$) (Supplementary Fig. 6A). Microbial richness (Faith's PD) dropped significantly in elderly ($P = 0.009$), but it was not significantly affected by GOS supplementation in adults ($P > 0.05$) (Supplementary Fig. 6B). Furthermore, the significant effects of GOS on relative abundance of *Bifidobacterium* and on microbial diversity were not significantly different between elderly and adults ($P \geq 0.337$).

3.3. Faecal metabolites

Acetate, propionate, butyrate, isobutyrate and succinate concentrations did not significantly differ between elderly and adults at baseline ($P \geq 0.125$) (Table 3). Furthermore, metabolite concentrations did not change after four weeks GOS versus placebo supplementation neither in elderly, nor in adults ($P \geq 0.520$).

3.4. Volatile organic compounds

The VOC-based statistical analysis reveled no major differences between elderly and adults at baseline with an AUROC of 0.62 in the validation set (Fig. 2A), indicating relatively small differences in exhaled breath profiles, which is in line with microbiota composition and faecal metabolite data. The visualisation of the data using the complete VOC-profile as shown in Fig. 2B showed no clear differences between adults and elderly.

With respect to the effect of treatment on the VOC profiles of exhaled breath, ASCA analysis did not show any significant differences between baseline and treatment for either of the two age

Table 2
Most abundant genera based on sequencing of the 16S rRNA gene, pre and post GOS and placebo intervention periods of the elderly (n = 20) and adults (n = 23).

	Elderly (n = 20)							Adults (n = 23)							*P*-value (elderly vs adults at baseline)
	GOS			Placebo			*P*-value	GOS			Placebo			*P*-value	
	Baseline	Week 1	Week 4	Baseline	Week 1	Week 4		Baseline	Week 1	Week 4	Baseline	Week 1	Week 4		
Blautia	0.091 [0.061; 0.105]	0.066 [0.036; 0.087]	0.079 [0.042; 0.101]	0.100 [0.073; 0.127]	0.073 [0.040; 0.109]	0.079 [0.054; 0.116]	1.000	0.092 [0.068; 0.138]	0.067 [0.046; 0.086]	0.056 [0.038; 0.077]	0.098 [0.075; 0.132]	0.082 [0.069; 0.101]	0.088 [0.065; 0.099]	0.415	0.797
Bifidobacterium	0.024 [0.011; 0.071]	0.294 [0.208; 0.400]	0.260 [0.080; 0.339]	0.027 [0.016; 0.046]	0.020 [0.007; 0.038]	0.042 [0.008; 0.071]	<0.001	0.070 [0.049; 0.144]	0.348 [0.208; 0.484]	0.304 [0.240; 0.517]	0.083 [0.027; 0.150]	0.083 [0.035; 0.149]	0.113 [0.045; 0.171]	<0.001	0.033
Faecalibacterium	0.065 [0.038; 0.077]	0.049 [0.035; 0.071]	0.058 [0.038; 0.08]	0.073 [0.033; 0.096]	0.066 [0.032; 0.111]	0.061 [0.024; 0.090]	0.979	0.096 [0.070; 0.117]	0.074 [0.047; 0.102]	0.070 [0.051; 0.089]	0.077 [0.050; 0.102]	0.092 [0.050; 0.115]	0.075 [0.057; 0.107]	0.214*	0.610
Unclassified genus within Lachnospiraeceae family	0.030 [0.016; 0.081]	0.030 [0.018; 0.051]	0.046 [0.023; 0.067]	0.043 [0.022; 0.083]	0.037 [0.016; 0.072]	0.044 [0.018; 0.051]	0.850	0.039 [0.029; 0.064]	0.041 [0.014; 0.05]	0.034 [0.012; 0.058]	0.038 [0.019; 0.058]	0.041 [0.023; 0.061]	0.062 [0.022; 0.082]	0.270	0.715
Subdoligranulum	0.037 [0.020; 0.075]	0.019 [0.010; 0.031]	0.022 [0.014; 0.037]	0.038 [0.019; 0.059]	0.032 [0.013; 0.053]	0.022 [0.012; 0.047]	0.213	0.036 [0.028; 0.046]	0.025 [0.013; 0.031]	0.016 [0.013; 0.025]	0.040 [0.022; 0.062]	0.034 [0.019; 0.041]	0.032 [0.018; 0.056]	0.476	0.857
Prevotella 9	0.001 [0.000; 0.073]	0.023 [0; 0.211]	0.002 [0; 0.077]	0.002 [0; 0.063]	0.001 [0; 0.111]	0.000 [0.000; 0.086]	0.873	0.000 [0.000; 0.008]	0.000 [0.000; 0.007]	0.000 [0.000; 0.005]	0.000 [0.000; 0.001]	0.000 [0.000; 0.017]	0.000 [0.000; 0.003]	0.407	0.486
Ruminococcus 2	0.024 [0.002; 0.081]	0.016 [0; 0.025]	0.012 [0; 0.06]	0.043 [0.003; 0.059]	0.024 [0.003; 0.058]	0.027 [0.004; 0.05]	0.298	0.004 [0.000; 0.060]	0.007 [0.000; 0.049]	0.012 [0.000; 0.028]	0.022 [0.000; 0.046]	0.007 [0.000; 0.037]	0.026 [0.000; 0.049]	0.469	0.606
Christensenellaceae R-7 group	0.010 [0.004; 0.036]	0.004 [0.000; 0.023]	0.011 [0.002; 0.022]	0.019 [0.005; 0.033]	0.017 [0.002; 0.039]	0.015 [0.003; 0.033]	0.083*	0.017 [0.006; 0.032]	0.008 [0.006; 0.014]	0.013 [0.003; 0.026]	0.022 [0.007; 0.040]	0.017 [0.010; 0.041]	0.021 [0.008; 0.028]	0.481	0.827
Bacteroides	0.014 [0.003; 0.043]	0.004 [0.001; 0.019]	0.007 [0.003; 0.031]	0.025 [0.006; 0.052]	0.028 [0.011; 0.062]	0.015 [0.002; 0.020]	1.000	0.023 [0.004; 0.035]	0.016 [0.006; 0.027]	0.012 [0.005; 0.038]	0.027 [0.009; 0.040]	0.017 [0.007; 0.058]	0.033 [0.022; 0.061]	0.523	0.771

GOS: galacto-oligosaccharides. Selection made based on average relative abundance (in baseline samples) above 2.5%. Data are expressed as median [IQR; *i.e.* Q1; Q3]. Sample sizes vary due to drop-outs and technical reasons. Elderly vs. adults at baseline of the first intervention period were compared by Mann–Whitney U tests. Within age groups, interventions were compared with variance components (random intercept) linear mixed models and corrected for baseline values. Analysis has been performed in all genera. For visibility, here only genera with average relative abundance >2.5% have been shown. *P*-values were corrected for multiple testing by false-discovery-rate (FDR) of Benjamini-Hochberg. * Significant before correction for multiple testing.

Table 3
Faecal metabolite concentrations (μmol/g dry content) before, during and after GOS and placebo interventions in elderly (n = 20) and adults (n = 23).

	Elderly (n = 20)							Adults (n = 23)							*P* - value (elderly vs adults at baseline)
	GOS			Placebo			*P*-value	GOS			Placebo			*P*-value	
	Baseline	Week 1	Week 4	Baseline	Week 1	Week 4		Baseline	Week 1	Week 4	Baseline	Week 1	Week 4		
Acetate	64.83 [40.74; 89.41]	80.01 [57.90; 120.72]	69.53 [45.84; 107.39]	81.71 [54.42; 139.90]	90.71 [55.56; 113.99]	73.8 [49.62; 140.69]	0.976	94.81 [72.89; 135.44]	76.98 [53.10; 154.52]	111.00 [58.20; 162.04]	77.90 [59.34; 112.65]	69.74 [36.03; 100.90]	85.90 [44.87; 112.30]	0.972	0.188
Propionate	20.79 [15.00; 33.61]	27.78 [16.73; 34.41]	20.91 [16.11; 31.01]	28.71 [17.03; 39.18]	26.23 [19.15; 32.73]	28.21 [17.20; 38.63]	0.737	31.66 [19.21; 42.52]	26.29 [18.38; 36.98]	27.64 [19.37; 38.75]	28.28 [19.13; 38.58]	25.50 [17.03; 42.88]	25.86 [15.75; 40.36]	0.654	0.125
Butyrate	14.74 [9.14; 20.45]	17.94 [9.13; 23.43]	15.28 [11.29; 19.13]	16.55 [10.72; 23.20]	16.17 [9.78; 23.59]	24.40 [8.05; 32.13]	0.270	24.93 [19.20; 41.89]	23.23 [16.79; 30.75]	22.14 [13.85; 36.07]	21.71 [14.05; 36.11]	22.55 [5.45; 37.06]	17.78 [11.71; 40.37]	0.951	0.125*
Isobutyrate	2.32 [1.31; 3.41]	2.315 [1.23 ;3.69]	3.30 [1.93; 3.64]	3.25 [1.88; 5.46]	3.15 [1.41; 4.48]	2.88 [2.01; 4.75]	0.560	2.64 [0.00; 4.26]	1.73 [0.00; 3.24]	2.82 [0.59; 4.48]	3.48 [1.59; 3.77]	2.24 [0.00; 3.63]	3.38 [0.68; 6.42]	0.288	0.487
Succinate	0.45 [0.10; 1.76]	0.18 [0.06; 0.36]	0.35 [0.13; 1.04]	0.285 [1.13; 1.92]	0.355 [0.06; 1.15]	0.18 [0.10; 0.61]	0.537	0.34 [0.00; 1.32]	0.59 [0.19; 3.11]	0.345 [0.06; 0.79]	0.18 [0.02; 0.83]	0.28 [0.00; 0.79]	0.23 [0.02; 1.40]	0.253	0.487

GOS: galacto-oligosaccharides. Data are expressed as median [IQR; *i.e.* Q1; Q3]. Sample sizes vary due to drop-outs and technical reasons. Lactate and formate concentrations were under the detection limit. Elderly vs. adults at baseline of the first intervention period were compared by Mann–Whitney U tests. Within age groups, interventions were compared with variance components (random intercept) linear mixed models and corrected for baseline values. *P*-values were corrected for multiple testing by false-discovery-rate (FDR) according to Benjamini-Hochberg. * Significant before correction for multiple testing ($P = 0.034$).

groups. The uRF score plots show no clear groupings between samples taken before and after the GOS and placebo four weeks intervention, indicating similarity in breath profiles (Fig. 3A-D).

3.5. Immune parameters

Cytokine production (IL-1β, IL-6, IL-8, IL-10, TNFα and IFN-γ) in plasma after 24 h PHA or LPS whole blood stimulations and serum CRP concentrations were not significantly different between elderly and adults at baseline (Fig. 4, all $P \geq 0.803$). Moreover, these parameters did not differ significantly between four weeks GOS and placebo supplementation neither in elderly nor in adults (Fig. 4, all $P \geq 0.964$). Overall, these results suggest that GOS did not significantly impact parameters of immune response in elderly or adults.

3.6. Parameters of systemic oxidative stress

Plasma MDA and UA concentrations as well as TEAC values did not differ significantly between elderly and adults at baseline (Fig. 5A-C) (all $P \geq 0.128$). However, baseline TEAC values corrected for UA plasma concentrations (Fig. 5D) were significantly higher in elderly compared with adults ($P < 0.001$). None of the markers significantly differed between subjects after four weeks GOS or placebo supplementation, neither in elderly, nor in adults (Fig. 5A-D, all $P \geq 0.236$). These observations indicate that GOS did not significantly alter parameters of systemic oxidative stress in elderly or in adults.

4. Discussion

We have shown that faecal microbiota composition differed significantly between age groups, with significantly lower relative abundance of *Bifidobacterium* in the elderly when compared with adults. Faecal and breath metabolites, as well as parameters of immune function and systemic oxidative stress did not show significant differences between elderly and adults. After four weeks of GOS supplementation, microbiota composition of both age groups changed significantly, accompanied by significant increase in *Bifidobacterium,* significant decrease in microbial diversity (in adults and in elderly) and microbial richness (in elderly). In the elderly and adults, faecal and breath metabolites, and parameters of immune function and systemic oxidative stress were not significantly different when comparing subjects that either received GOS or placebo intervention.

Microbiota perturbations in elderly have been shown to be more pronounced in elderly with impaired health status (*e.g.* frailty) [5]. In the current study, the overall microbiota composition differed significantly between prefrail elderly and healthy adults with around 4% of the total microbiota variation explained by subject age group and *Bifidobacterium* as the main contributer to this difference. Consistently, Claesson et al. [39] found that elderly subjects (aged 64–102 yrs) did cluster based on residence location, with microbial diversity being lowest in people staying long-term in residential care, being indicative for increased comorbidity and frailty. In the current study, we included community dwelling elderly without major comorbidities and applied the widely used Fried criteria (14) to identify physically 'prefrail' elderly. Others using the Rockwood Frailty index (including a broader range of deficits) or the Barthel Index (assessing performance in activities of daily living), did find a negative association between microbial richness and frailty level [40,41]. In the current study, although no negative correlation between frailty score and microbial diversity has been observed, the significantly lower faecal bifidobacterial abundance in prefrail elderly compared with

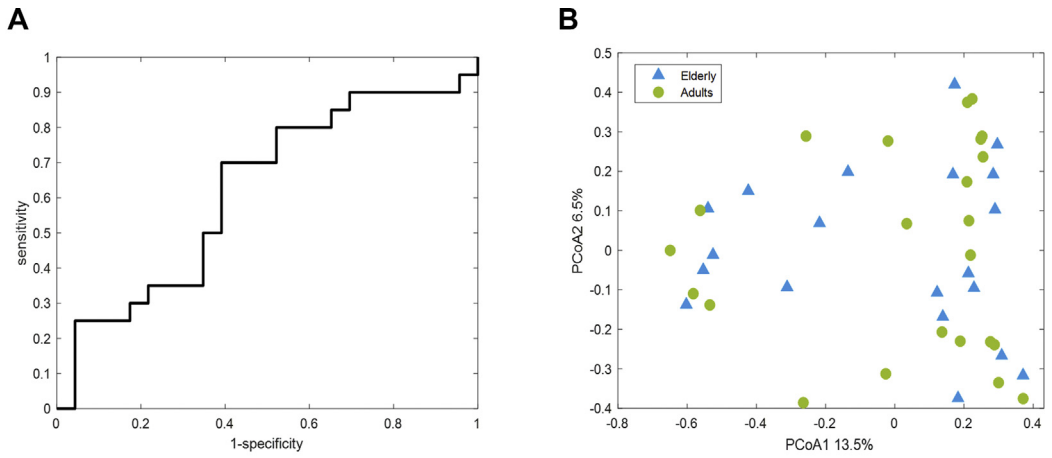

Fig. 2. (A) Receiver operating characteristic curve performed on the validation set, with area under the curve = 0.62. (B) unsupervised random forest score plot performed on the VOC profiles obtained from participants at baseline, showing no clear groupings in exhaled breath profiles between 20 elderly (blue triangles) and 23 adults (green dots). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

healthy adults is in line with previous studies [42–44]. In the present study, anticoagulation, antihypersentives and statins were allowed on a stable dose as we did not expect them to interfere with the intervention, which was indeed confirmed by our analyses. Although officially excluded, analyses revealed that two elderly subjects were using PPIs. However, excluding these from the analyses did not impact our main findings (data not shown). In addition, as found previously [12,45–47], in the current study, four weeks GOS supplementation (15 g/day) resulted in a significant increase in faecal bifidobacteria abundance and differences in

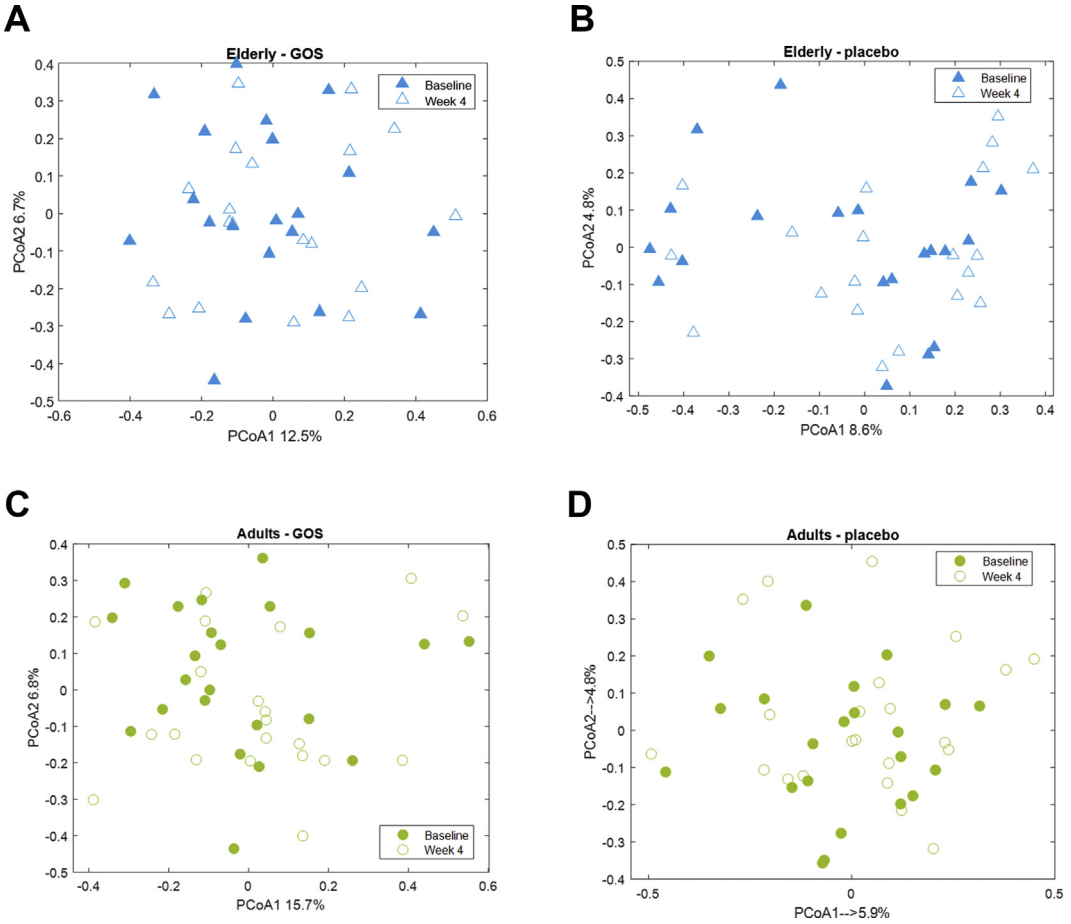

Fig. 3. Unsupervised random forest score plots based on the complete VOC profiles (A) found in the exhaled breath of 20 elderly at baseline and 4 weeks post-GOS intervention; (B) found in the exhaled breath of 20 elderly at baseline and post-placebo intervention; (C) found in the exhaled breath of 23 adults at baseline and 4 weeks post-GOS intervention; (D) found in the exhaled breath of 23 adults at baseline and 4 weeks post-placebo intervention. Sample sizes vary due to drop-outs and technical reasons.

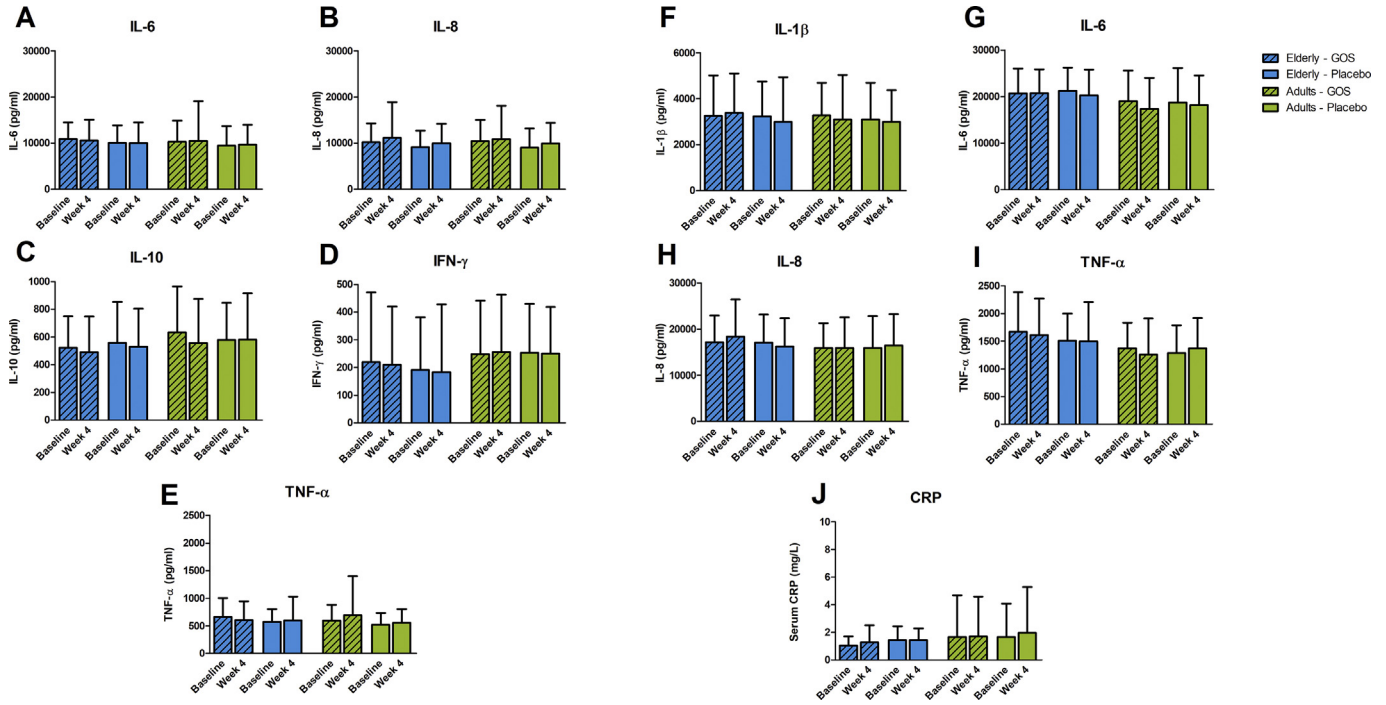

Fig. 4. Whole blood cytokine production after 24 h 10 μg/ml PHA (A–E) or 10 μg/ml LPS (F–I) stimulation and serum CRP concentrations (J), at baseline and after 4 weeks of GOS (striped) and placebo (unstriped) intervention in 20 elderly (blue bars) and 24 adults (green bars). Values are presented in bars with mean and SD. Sample sizes vary slightly due to drop-outs and technical reasons. Elderly vs. adults at baseline of the first intervention period were compared by an independent samples t-test. Within age groups, interventions were compared with variance components (random intercept) linear mixed models and correction for baseline values. P-values were corrected for multiple testing by false-discovery-rate (FDR) of Benjamini-Hochberg. CRP, C-reactive protein; LPS, lipopolysaccharide; PHA, phytohemagglutinin-M. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

overall faecal microbiota composition of adults and elderly. Moreover, in the present study, the food intake data of recruited subjects showed no changes in micronutrient and micronutrient intake over time, suggesting the contribution of GOS intake to the observed changes in microbiota (*i.e.* in bifidobacteria). Despite differences in bifidobacterial abundance, faecal metabolites did not show significant differences neither between elderly and adults, nor between subjects consuming either GOS or placebo supplements. It should be noted that these findings should be interpreted with care, because SCFAs are readily absorbed and faecal metabolites do not reflect metabolite production of the proximal colon (*i.e.* assumed site of GOS fermentation).

Previous studies showed VOCs to be linked to both the microbial activity [48,49] as well as host pathophysiological processes such as inflammation [50,51]. In the current study, however, the analyses of exhaled breath revealed no significant differences in VOCs between elderly and adults. This is in line with a previous study of our group [15].In addition, no significant changes in VOC profiles were found after four weeks of GOS supplementation both in elderly as well as in young adults. It has been suggested that diet may impact the generation of compounds found in exhaled breath [52–54]. GOS is a dietary fibre, of which we assumed that due to changes of the intestinal microbiota activity, VOC profiles could potentially be altered. However, no significant effects of GOS on faecal metabolites or VOCs in exhaled breath were found. A recent study by Drabińska et al. [55] demonstrated moderate changes in urine VOC patterns after 12 weeks supplementation of oligofructose-enriched inulin (*i.e.* Synergy 1). It should be noted, however, that study was performed in children suffering from celiac disease.

As several studies have observed that immunosenescence is common in elderly, and the intestinal microbiome plays a role in intestinal immune functioning, we have evaluated cytokine responses after *ex vivo* PHA and LPS stimulation of whole blood for 24 h. We found that LPS-stimulated whole blood IL-1β, IL-6, IL-8 and TNF-α concentrations were not significantly different between elderly and adults. Our findings contrast with those of Bruunsgaard et al. [56] who found that LPS-stimulated IL-1β and TNF-α, but not IL-6 levels, were lower in a mixed group of healthy and comorbid elderly (80–81 yrs, n = 168) compared to healthy young adults (19–31 yrs, n = 91). Our results that PHA-stimulated IL-6, IL-8, IL-10, IFN-γ and TNF-α concentrations did also not differ significantly between elderly and adults, are in line with previous findings on IL-6 production after PHA stimulation in isolated peripheral blood mononuclear cells (PBMC) [57,58]. We also evaluated serum CRP as frailty has been associated with inflammation [59]. CRP levels were not significantly different between elderly and adults in our study. Taken together, our findings point towards a relatively healthy elderly population with a preserved immune response, despite being defined as physically prefrail by the Fried criteria [14].Four weeks of GOS supplementation (*i.e.* 21.6 g/day of Biotis™ GOS Powder) did not significantly impact cytokine production by LPS or PHA whole blood stimulations, neither in elderly nor in adults. Interestingly, in the study of Vulevic et al. [12] 5.5 g/day of GOS intake for five weeks in healthy elderly (64–79 yrs) resulted in significantly decreased LPS-stimulated production of IL-6 and TNF-α in PBMC, pointing towards anti-inflammatory properties of this relatively low dose of GOS. On the other hand, IL-1β and IL-8 production were not affected in that study [12]. Data on habitual dietary intake and other lifestyle factors were not reported, and can therefore not be compared. Further, apart from some small methodological differences, a clear biological explanation for the contrasting findings cannot be given. We speculate that these differences might be cohort-specific based on a recent study that showed that

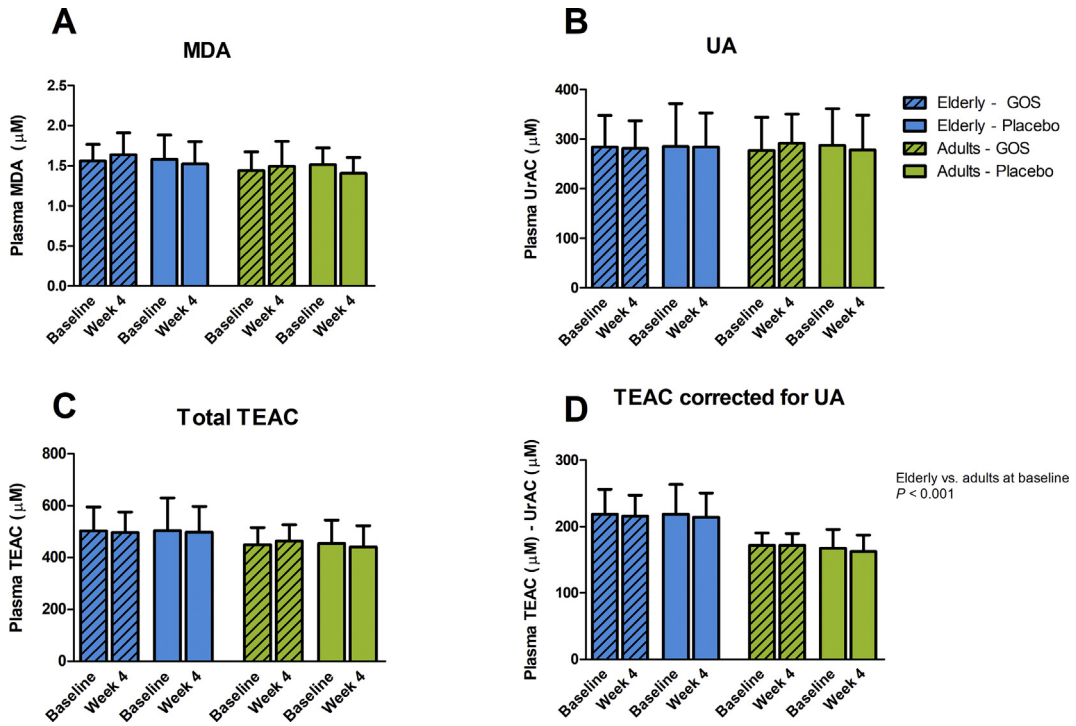

Fig. 5. Plasma concentrations of MDA (A) and UA (B), total TEAC values (C), and TEAC values corrected for UA plasma concentrations (D), at baseline and after 4 weeks GOS (striped) or placebo (unstriped) intervention in 20 elderly (blue bars) and 24 adults (green bars). Values are presented in bars with mean and SD. Sample sizes vary due to drop-outs and technical reasons. Elderly vs. adults at baseline of the first intervention period were compared by an independent samples t-test. Within age groups, interventions were compared with variance components (random intercept) linear mixed models and correction for baseline values. P-values were corrected for multiple testing by false-discovery-rate (FDR) of Benjamini-Hochberg. MDA, malondialdehyde; TEAC, trolox equivalent antioxidant capacity; UA, uric acid. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

differences between cohorts with similar selection criteria can already have a huge impact on observations [60].

As immune function is associated with oxidative stress [8], and elevated oxidative stress levels and lower antioxidant capacity have been reported in elderly [61–65], we also determined the concentrations of the lipid peroxidation marker MDA and the antioxidant capacity of plasma (TEAC) in elderly and adults. Both were not significantly different between elderly and adults.

In addition, we showed that four weeks GOS intervention did not significantly alter markers of oxidative stress and antioxidant capacity in either of the age groups. Interestingly, when we corrected TEAC values (*i.e.* total antioxidant capacity) for the most abundant antioxidant in plasma, UA, the antioxidant capacity was found to be even higher in elderly compared with adults. However, dietary intake (*i.e.* polyphenol intake) did not differ significantly between age groups.

Our data indicate that the included prefrail elderly turned out to be relatively healthy, with no significant baseline differences between elderly and adults, and lack of a GOS effect on faecal metabolites as well as parameters of immune function and systemic oxidative stress. The Fried frailty criteria refer to physical (pre) frailty, and do not necessarily select for subjects with an altered intestinal microbiota or immune-related deficits. Further, we have shown that GI symptom scores and stool frequency (including frequencies of hard stools and loose stools) were not significant different between GOS and placebo intervention neither in elderly, nor in adults. Therefore, the addition of a relatively high dose of GOS (15.0 g/day) was well-tolerated, and these findings are in line with other dietary fibre intervention studies in comparable populations [66,67]. Moreover, compliance as determined by returned empty sachets was high (95.0% in adults and 97.6% in elderly), although we acknowledge this is a subjective measure.

In conclusion, we showed that bifidobacteria were lower in prefrail elderly compared with adults, but faecal and breath metabolites, as well as parameters of immune function and oxidative stress were not significantly different. It remains to be determined what the potential consequences of low bifidobacterial counts in *e.g.* prefrail elderly are for the longer term. By performing longitudinal studies, it can be investigated whether low bifidobacterial abundance will be a risk factor for impaired health functions, such as frailty. Further, in this well-controlled study, four weeks GOS supplementation increased bifidobacteria and decreased microbial diversity, but did not affect faecal and breath metabolite concentrations, immune function and oxidative stress, based on the parameters measured. Future intervention studies aiming to improve immune-related health status should select more vulnerable subgroups of (frail) elderly, preferably by using biomarkers and/or based on the outcome parameters.

Author contributions

Ellen Wilms: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – Orginal draft, Visualization, Project administration. Ran An: Conceptualization, Methodology, Formal analysis, Data curation, Writing – Orginal draft, Visualization. Agnieszka Smolinska: Formal analysis, Resources, Data curation, Writing – Orginal draft, Visualization. Yala Stevens: Investigation, Writing – Review & Editing. Antje R. Weseler: Resources, Writing – Review & Editing. Montserrat Elizalde: Formal analysis. Marie-José Drittij: Formal analysis. Athanasia Ioannou: Formal analysis, Writing – Review & Editing. Frederik J. van Schooten: Resources, Writing – Review & Editing. Hauke Smidt: Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision. Ad A.M. Masclee: Conceptualization,

Methodology, Resources, Writing – Review & Editing, Supervision. Erwin G. Zoetendal: Conceptualization, Methodology, Resources, Writing – Orginal draft, Supervision, Funding acquisition. Daisy M.A.E. Jonkers: Conceptualization, Methodology, Resources, Writing – Orginal draft, Supervision, Funding acquisition.

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Conflict of interest

The funders had no role in the study design, data collection, data analyses, or manuscript related decisions. YS is an employee of BioActor BV. DJ was in part supported by a Grant Top Knowledge Institute (Well on Wheat). All other authors disclose no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2020.12.034.

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