

RESEARCH ARTICLE

Lactobacillus casei Shirota Supplementation Does Not Restore Gut Microbiota Composition and Gut Barrier in Metabolic Syndrome: A Randomized Pilot Study

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Abstract

Metabolic syndrome is associated with disturbances in gut microbiota composition. We aimed to investigate the effect of *Lactobacillus casei* Shirota (*LcS*) on gut microbiota composition, gut barrier integrity, intestinal inflammation and serum bile acid profile in metabolic syndrome. In a single-centre, prospective, randomised controlled pilot study, 28 subjects with metabolic syndrome received either *LcS* for 12 weeks ($n = 13$) or no *LcS* ($n = 15$). Data were compared to healthy controls ($n = 16$). Gut microbiota composition was characterised from stool using 454 pyrosequencing of 16S rRNA genes. Serum bile acids were quantified by tandem mass spectrometry. Zonulin and calprotectin were measured in serum and stool by ELISA. *Bacteroidetes/Firmicutes* ratio was significantly higher in healthy controls compared to metabolic syndrome but was not influenced by *LcS*. *LcS* supplementation led to enrichment of *Parabacteroides*. Zonulin and calprotectin were increased in metabolic syndrome stool samples but not influenced by *LcS* supplementation. Serum bile acids were similar to controls and not influenced by *LcS* supplementation. Metabolic syndrome is associated with a higher *Bacteroidetes/Firmicutes* ratio and gut barrier dysfunction but *LcS* was not able to change this. *LcS* administration was associated with subtle microbiota changes at genus level.

Trial Registration

ClinicalTrials.gov [NCT01182844](https://clinicaltrials.gov/ct2/show/study/NCT01182844)

Competing Interests: The authors have declared that no competing interests exist.

Introduction

The obesity epidemic is a challenging threat to public health in the 21st century. The World Health Organisation states that by 2014 approximately 1.9 billion adults are overweight (BMI 25–29.9 kg/m²), and more than 600 million are obese (BMI 30 kg/m² or more). [1] Obesity and insulin resistance are major risk factors for the development of metabolic syndrome (MetS), type 2 diabetes mellitus (T2DM), and conditions such as cardiovascular morbidity and mortality. [2, 3]

The pathogenesis of obesity is multifactorial and is seen as interplay between individual phenotype and environmental factors. However, recent preclinical and clinical studies show an important influence of the intestinal microbiota on obesity and associated metabolic disorders (MetS, T2DM, metabolic liver diseases, cardiovascular diseases). [4] The cecum microbiota of obese mice was found to be significantly different with a higher prevalence of *Firmicutes* and a corresponding lower prevalence of *Bacteroidetes* (lower *Bacteroidetes/Firmicutes* ratio) in obese compared to lean mice. [5] This finding of altered gut microbiota was then confirmed and extended to humans. [6] However, others did not find this association in obesity or MetS. [7–10] Further studies have shown an association of gut microbiota changes with insulin resistance and diabetes. [11, 12] This suggests that the microbiota might be involved in the pathogenesis of obesity, insulin resistance and T2DM, possibly by having an impact on gut barrier integrity and inflammation. [4] Also, hydrophobic bile acids have been proposed as a novel mechanism for high fat diet induced gut barrier dysfunction. [13, 14] Probiotic interventions have been shown to be effective in modulating gut barrier integrity and gut microbiota in animals and thereby modulating chronic inflammation and metabolic disorders in animal models. [15, 16] Despite several mechanistic studies and encouraging results in animals [17, 18] interventional data on probiotics use in humans with MetS are rare. [19]

The aim of our study was to investigate the effect of *Lactobacillus casei* Shirota (LcS) on gut microbiota composition, gut barrier integrity, intestinal inflammation and the serum bile acid profile in MetS.

Patients and Methods

Patients and Controls

The study was conducted according to the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Committee of the Medical University of Graz (20–037 ex 08/09). The study was registered at ClinicalTrials.gov (NCT01182844). Due to an unexpected organizational delay in the registration process of the study the initial release of the protocol in ClinicalTrials.gov took place after the first patient was randomized. The authors confirm that they have not performed and are not conducting any other trials with this intervention (*Lactobacillus casei* Shirota). Written informed consent was obtained from all subjects. The primary and parts of the secondary outcomes were already published [20, 21]. The gut microbiome analysis was delayed due to lack of funding. The Ethics Committee of the Medical University was informed about the delay in analysis and about all changes in the study protocol. These changes were methodological changes since the methods for gut microbiome analysis and gut permeability analysis have advanced since the start of the study. Furthermore the analysis of bile acids has been added to the protocol and was approved by the Ethics Committee of the Medical University.

According to the modified NCEP-ATP-III-guidelines [22] patients with MetS were identified from the outpatient clinic at the Division of Endocrinology and Metabolism at the Medical University of Graz. Patients treated with antibiotics within the previous 7 days, with current

anti-hyperglycemic treatment, any immunomodulatory therapy 1 month prior to study entry, concomitant use of pre-, pro-, or synbiotics, inflammatory bowel disease (Crohn's disease, ulcerative colitis) or celiac disease or those with clinical signs of infectious diseases were excluded from participation. We performed a single-centre, permuted-block randomised controlled 12 weeks prospective intervention trial. Patients were randomised to receive either food supplementation with a milk drink containing LcS (3 bottles a day, à 65ml, containing LcS at a concentration of 10^8 /ml, Yakult light®[®], Yakult Austria, Vienna, Austria) for twelve weeks (n = 13, LcS group) or no intervention (n = 15, standard therapy group).

Patients were randomised with the “Randomizer®” software using permuted blocks (Institute for Medical Informatics, Statistics and Documentation, Medical University of Graz, Austria). All patients were advised to consume no other probiotic supplements during the study period and received a list of probiotic products available in Austria, which they had to avoid for the study period. Participants were also advised not to change their diet and physical activity pattern while being in the study. A food frequency questionnaire was used to confirm unchanged diet habits. [20] Subjects of the LcS group were provided with a pack of the milk drink every two weeks. At these time points intervention adherence was assessed.

Stool and serum samples were collected at baseline and after 12 weeks. Stool samples from 16 healthy, lean controls without evidence of metabolic syndrome were used as a comparison for gut microbiota analysis and zonulin levels. For stool calprotectin established reference ranges from the kit description were used. Serum samples from 11 healthy, lean controls were used to compare bile acid profiles, zonulin, and calprotectin levels in serum.

DNA-isolation, 454 library preparation and sequencing

Stool samples were immediately frozen and stored at -80°C until semi-automated DNA isolation. Approximately 175mg of stool was homogenized in MagnaLyser Green Bead tubes by using the MagnaLyser Instrument (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. Total genomic DNA was isolated with the MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) in a MagNA Pure LC 2.0 Instrument (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. Enzyme cocktail II (Roche Diagnostics, Mannheim, Germany) with 100µg lysozyme (Karl Roth GmbH, Karlsruhe, Germany) per 100µl sample was used according to manufacturer's instructions.

The 16S rRNA gene was amplified using FLX 454 one way read (Lib-L kit, Primer A, Primer B, Roche 454 Life Science, Branford, CT, USA) (S1 Table) fusion primers with the template specific sequence F27—AGAGTTTGATCCTGGCTCAG and R534—ATTACCGCGGCTGCTGGC targeting the V1-V3 hypervariable regions [23, 24] as described previously in Kump et al. 2013. [25]

Zonulin and calprotectin

A ready-to-use solid-phase sandwich ELISA (Immundiagnostik AG, Bensheim, Germany) was used to detect zonulin (zonulin Serum or Stool ELISA) and calprotectin (PhiCal® calprotectin Serum or Stool ELISA) in serum and stool samples. The tests were performed according to the manufacturer's instructions. For stool sampling the Stool Sample Application System (Immundiagnostik AG, Bensheim, Germany) was used according to the manufacturer's manual.

Bile acids

All bile acids (cholic acid, CA; deoxycholic acid, DCA; chenodeoxycholic acid, CDCA; lithocholic acid, LCA; ursodeoxycholic acid, UDCA) were assessed as unconjugated acids and as taurine and glycine conjugates using a tandem mass spectrometry method as described

previously. [26] All sub-fractions of bile acids (free acids and their corresponding conjugates) were analysed by three different multiple-reaction monitoring experiments within one HPLC run. HPLC was performed on a reversed-phase (C18) column that used a methanol/water gradient for chromatographic solution of isobaric bile acids. Deuterated internal standards and correlation of peak area ratios in linear regression were used for quantification of all sub-fractions of bile acids.

Data analysis. Raw sequencing data generated on the Genome Sequencer FLX system were de-noised with Acacia 1.52. Afterwards reads were pre-processed with several quality parameters to trim primer and barcode sequences and filter low quality reads according to suggestions described in Huse et.al. [27]. Data analysis was performed in QIIME 1.7.0 [28] and included following steps: clustering of high quality reads into operational taxonomic units (OTUs) using UCLUST [29] and similarity of 0.97; taxonomy assignment of the OTUs with RDP classifier [30] and confidence score of 0.8 based on GreenGenes [31] 16S rRNA database; representative sequence alignment with PyNAST [32]; detection and removal of chimeric sequences with ChimeraSlayer; [33] generating a phylogenetic tree with FastTree [34]. Additionally singletons (OTUs with one read present only in one sample) were removed to avoid overestimation of the sample richness and diversity. Finally, the resulting OTU table and accompanied phylogenetic tree were further used for calculation of alpha and beta diversity. Alpha diversity indices were determined with the R statistical programming language extended with Vegan and BiodiversityR community ecology packages whereas beta diversity was done in QIIME involving data rarefaction to the smallest sample size and selecting two distance measurements, weighted UniFrac and Bray-Curtis distance for the Principal Coordinates Analysis (PCoA) diagrams and between samples comparisons. Testing whether groups of the samples were significantly different was assessed with the non-parametric MANOVA method implemented in the Vegan package (Adonis) or with Student's t-Test and ANOVA as implemented in R environment.

We searched for *Lactobacilli*, because sequencing only a fragment of the 16S rRNA gene does not allow us to determine species with a high confidence.

Searching for *Akkermansia* was done using taxonomic information in the OTU table.

All other statistical analyses were performed using SPSS 18.0 software (SPSS Inc, Chicago). The Mann-Whitney-U test or the unpaired student's t-test were used for the comparison of differences between groups and the paired student's t-test or the Wilcoxon signed-rank test for the before and after treatment measurements, as appropriate for normally and not-normally distributed variables, respectively. Differences with a p-value below 0.05 were considered statistically significant and for multiple tests we used Benjamini & Hochberg correction.

Results

Patients

Thirty-five subjects were screened for the study between January and August 2010; 30 patients were finally included, whereof 28 finished the study (2 dropped out due to withdrawal of informed consent). Five patients did not fulfill the inclusion criterion of fasting glucose above 100 mg/dl at the day of screening any more. Thirteen patients were randomized to the probiotic group and 15 to the standard therapy group (Fig 1). Baseline characteristics of patients with MetS and a healthy control group are shown in Table 1.

Gut microbiota

We generated 539,934 raw sequences with a mean length of 362bp. After de-noising and quality filtering, 390,021 reads (mean length 393bp) remained for downstream analysis. Chimeric

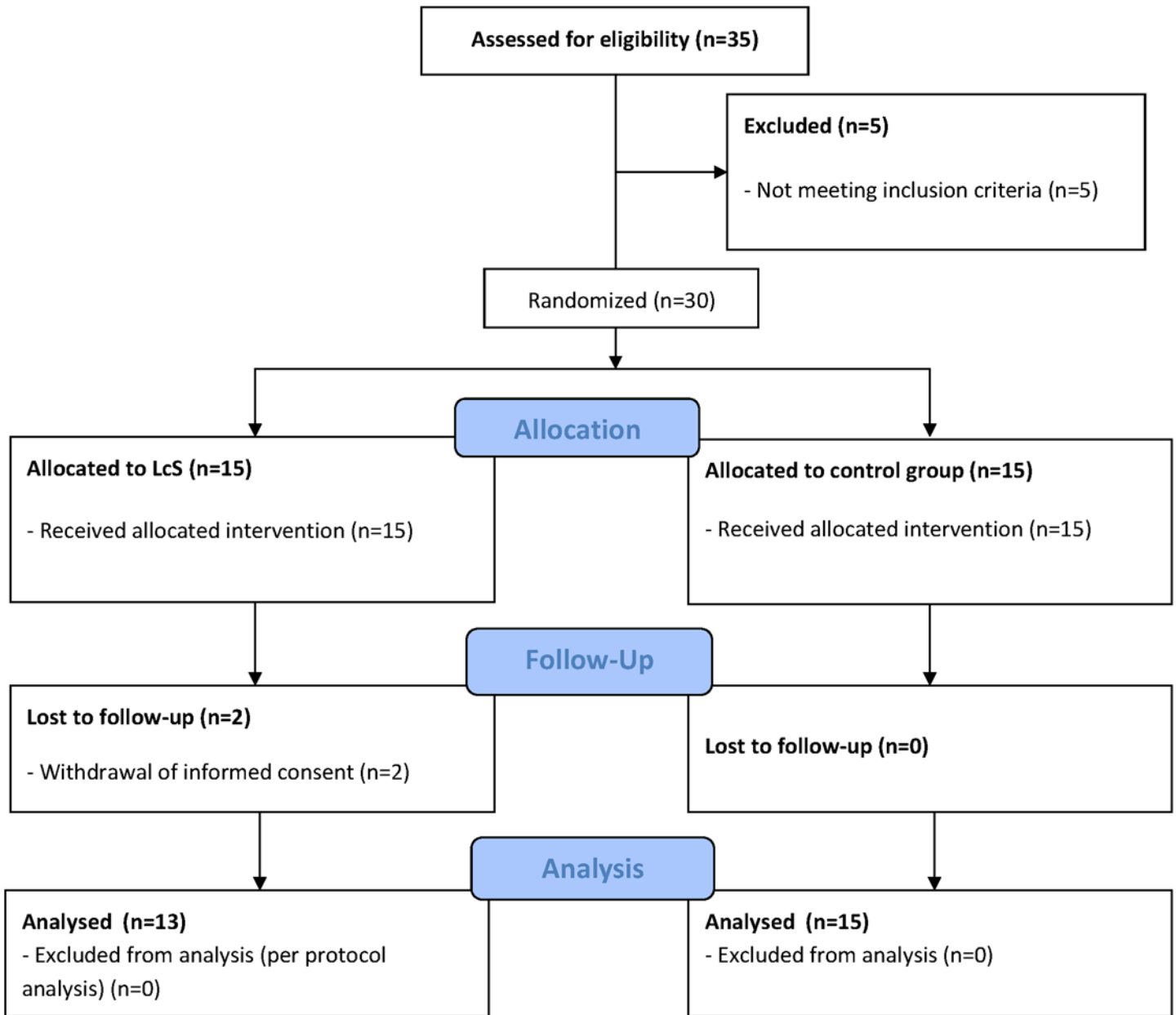


Fig 1. Flow diagram of the study progress.

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sequences (18.5%) and singletons (2.71%, reads occurring only once in a single sample) were removed and not used for further analysis. The average number of reads per sample was 5,208 (SD 1,786; range 1,716–11,252). Rarefaction curves confirmed that sequencing effort was not sufficient to cover all rare taxa, but still the curve trends were approaching saturation, and did not show any specific group dependent characteristics.

Comparison between MetS patients and healthy controls. *Bacteroidetes/Firmicutes* ratio was significantly lower in patients with MetS (median: 0.75; quartiles: 0.44–1.00; $p < 0.0001$) compared to healthy controls (median: 1.77; quartiles: 0.89–2.42; Fig 2A). We could recognize two groups of samples, where *Bacteroides* (68%) or *Prevotella* (15%) was most abundant as well

Table 1. Patient characteristics. Data are given as mean±SD.

	LcS (n = 13)		Standard therapy (n = 15)		Healthy (n = 16)
	base	EOS	base	EOS	
Sex (female/male)	4/9	4/9	6/9	6/9	7/9
Age (years)	51±11	51±11	55±9	55±9	25±4***
Height (cm)	175±8	175±8	169±8	169±8	172±8
Weight (kg)	109±15	108±17	91±14**	91±15	69±11***
Blood pressure systolic (mmHg)	148±19	142±16	147±18	139±11	n.a.
Blood pressure diastolic (mmHg)	95±12	92±12	94±18	88±9	n.a.
Body mass index (kg/m ²)	35±5	35±6	32±4*	32±4	23±3***
Waist circumference (cm)	113±12	112±12	106±8	106±9	76±8***
Total cholesterol (mmol/l)	220±69	219±59	209±43	211±34	4.9±1
High density lipoproteins (mmol/l)	43±17	40±16	47±19	42±12	1.6±0.3*
Low density lipoproteins (mmol/l)	128±49	132±45	119±27	126±29	2.7±0.9
Triglycerides (mmol/l)	214±169	202±123	170±106	159±66	1.2±0.5*

*p<0.05

**p<0.01

***p<0.001 compared to the other groups at baseline

n.a. not available; EOS: end of study; base: baseline; healthy: healthy controls.

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as different other genera making the rest of the samples (17%). No statistical differences in the *Bacteroides* and *Prevotella* groups' distribution between patients and controls were detected. No significant differences in UniFrac (p = 0.70) or Bray-Curtis (p = 0.48) distances between baseline and end of therapy were found. Evenness and diversity (Shannon and Simpson Index) of gut microbiota in MetS showed similar distribution compared to healthy controls (Table 2).

Akkermansia, a possibly relevant genus in the pathophysiology of MetS, was detectable in higher abundance in only one individual in the standard therapy group at baseline and at the end of the study (21.40% and 24.28%, respectively).

Effect of supplementation of LcS in MetS. Variations (UniFrac distances) in microbiota composition found in the LcS group between baseline and end of the study were similar to variations in the standard therapy group. Diversity of gut microbiota in MetS was not influenced by LcS supplementation. We also could not detect any significant changes in microbiota between the LcS and standard therapy group at any time point using Adonis multivariate analysis. Weighted UniFrac PCoA plots showed no clear separation of the groups between the two time points and in comparison with the healthy controls (Fig 2B).

By looking at the most abundant genera however (present with more than 1% in at least 50% of the samples) we found a significantly increased proportion of *Parabacteroides* at the end of the study when compared to baseline in the LcS group (p = 0.002). (Fig 2C)

Lactobacillus genus was not detectable with our method.

Gut wall integrity and inflammation

Zonulin and calprotectin were studied as markers of gut barrier disruption and intestinal inflammation. Serum levels of zonulin and calprotectin were not elevated in patients compared to healthy controls. However, zonulin and calprotectin were significantly higher in stool samples of patients compared to controls (p<0.001) or compared to the median of healthy controls

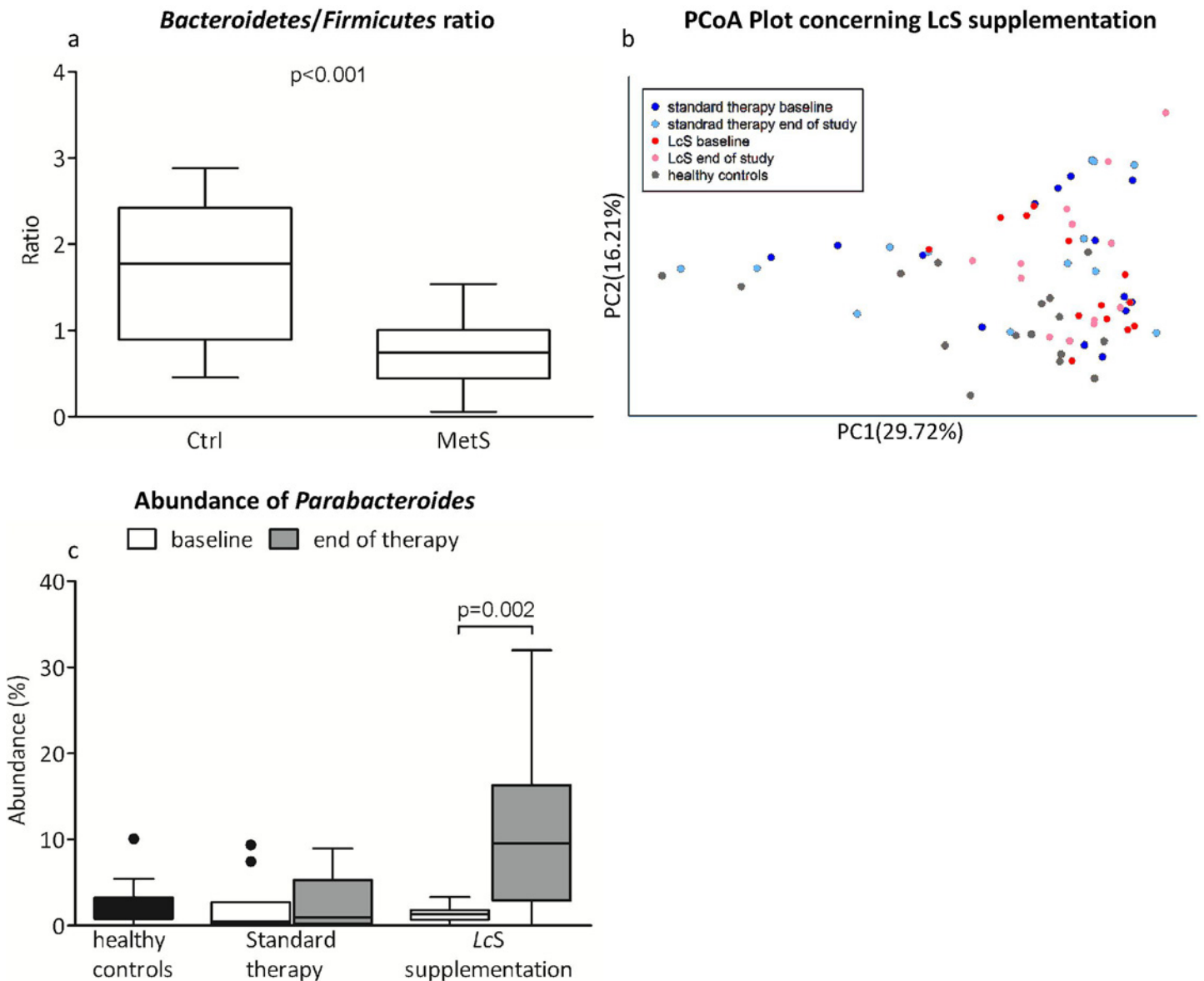


Fig 2. Gut microbiota composition in MetS patients and controls. *Bacteroidetes/Firmicutes* ratio (a) PCoA Plot (Weighted UniFrac, b) and abundance of *Parabacteroides* (c) concerning LcS supplementation.

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from the kit description ($p = 0.017$). Both zonulin and calprotectin levels were not influenced by LcS administration. Baseline data for zonulin and calprotectin are shown in [Table 3](#).

Table 2. Evenness and diversity of gut microbiota in MetS and healthy controls. Data are given as median (quartiles).

	MetS patients (n = 28)	Healthy controls (n = 16)	p-value
J-evenness	0.62 (0.56–0.66)	0.65 (0.59–0.66)	0.238
Shannon	3.8 (3.5–4.2)	3.9 (3.5–4.1)	0.839
Simpson	0.91 (0.88–0.95)	0.92 (0.87–0.95)	0.985

MetS: metabolic syndrome.

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Table 3. Zonulin and Calprotectin concentrations in serum and stool of MetS patients and controls. Data are given as median (quartiles).

	MetS patients (n = 28)	Normal range#
Serum zonulin (ng/ml)	45 (39–50)	52 (47–60)
Serum calprotectin (ng/ml)	555 (432.5–771.3)	570 (490–1050)
Stool zonulin (ng/ml)	75 (55–105)	31 (26–38) ***
Stool calprotectin (µg/ml)	29 (14.1–102.5)	25*

Data from 11 healthy controls for serum parameters, data from 25 healthy controls for zonulin in stool, median of healthy controls from the kit description for calprotectin

*** p<0.001 compared to MetS

* p = 0.017 compared to MetS.

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Bile acids

Total bile acids in serum were within the normal range in 26 out of 28 patients. In two patients, total bile acids were slightly elevated (8.2 and 6.9µmol/l; upper limit of normal: 6.5µmol/l). No difference in individual bile acids or total bile acids was found between patients with MetS and healthy controls. Proportions of primary (cholic acid, CA; chenodeoxycholic acid, CDCA) and secondary (deoxycholic acid, DCA; lithocholic acid, LCA; ursodeoxycholic acid, UDCA) as well as proportions of taurine- or glycine conjugated bile acids were unaltered in MetS when compared to healthy controls. Absolute and relative amounts of individual bile acids, primary, secondary or proportions of taurine- or glycine conjugated bile acids did not change after consumption of LcS for 12 weeks. No compelling evidence for a correlation between serum bile acids and markers of gut barrier disruption and inflammation was found.

Discussion

We found that the *Bacteroidetes/Firmicutes* ratio, markers of gut barrier disruption and inflammation of patients with MetS differ significantly from healthy controls. Supplementation with LcS increased the abundance of *Parabacteroides* but did not influence any other markers investigated in this study. Serum bile acid profile in MetS was not different to healthy controls.

We could confirm previously published findings of increased *Firmicutes* and decreased *Bacteroidetes* in obesity and MetS. [35–38] The gut microbiota has been shown to influence energy extraction from diet, regulation of lipogenesis and innate immunity and therefore plays an important role in the pathogenesis of MetS. [39] We could find the presence of two genera (*Bacteroides* and *Prevotella*) remaining stable over the study period and not influenced by LcS. This confirms previous findings, showing no changes of the two genera, that other authors defined as enterotypes, during a 6-months controlled diet intervention. [9, 40]

An interesting species—*Akkermansia muciniphila*—has been associated with weight gain, insulin resistance and inflammatory changes. [41, 42] In our study we were not able to reproduce this finding.

The supplementation with LcS led to a significant increase in *Parabacteroides* (phylum *Bacteroidetes*). From our data we cannot explain this finding, we can only hypothesize that LcS might lead to ecological rearrangements in gut microbiota leading to this increase in *Parabacteroides*. *Parabacteroides* derive energy mainly from fermentation from carbohydrates or proteins, however the amount of carbohydrates in the milk drink is probably too small to be responsible for this increase. It is unclear, if an increase of *Parabacteroides* is beneficial. A shift from *Firmicutes* towards *Bacteroidetes* could be seen as beneficial in metabolic disorders [43]

and dietary supplementation of resistant starch has been shown to increase *Parabacteroides* [44], whereas a reduction was associated with recurrence in Crohn's disease. [45] Contrary, in non-alcoholic steatohepatitis, a higher abundance of *Parabacteroides* was observed. [46] Since we previously published that supplementation with LcS did not influence clinical and biochemical parameters of glucose metabolism, inflammation and innate immune response [20, 21], the relevance of our observation is unclear and cannot be answered by our study design. For more detailed information concerning abundance of gut microbiota see [S2 Table](#).

We have chosen to study *Lactobacillus casei* Shirota for several reasons. This commercially available milk drink delivers a relatively high bacterial number in a relatively small volume. LcS has been proven to survive the passage through the stomach and is still present in the lower intestinal tract [47–49]. Within previous studies LcS appeared to be effective in modulating natural killer cell [50] and neutrophil function [51]. Previous studies detected LcS in stool (both by PCR and culture) already after 7 days of ingestion of an LcS containing milk drink [52–54]. In those studies, different milk drinks with 5–15 times higher daily concentrations of LcS compared to our study were used. LcS was also detected in stool of European subjects, taking the same product we used in our study, using culture techniques for LcS detection. [55, 56] With the methodology we used we could not detect an increase in *Lactobacillus* genera by probiotic supplementation in stool samples. This is not unexpected considering the number of reads, the concentration of the product (6.5×10^9 per day) and the dilution of the probiotic milk drink in the gut with a bacterial count of about 10^{14} cells. Therefore methodological limitations (limited number of reads per sample), differences in patient population and/or in the product can explain the different findings between our study and previous data.

In animal models increased gut permeability is associated with increased translocation of bacterial products and contributes to insulin resistance. [17] In a previous study we could show that subjects with MetS have increased gastroduodenal and small intestinal permeability compared to healthy controls. [20] Zonulin is the only physiological mediator known to regulate intestinal permeability reversibly by modulating intercellular tight junctions [57–59] and a positive correlation between zonulin, obesity and insulin resistance has been found recently. [60] Calprotectin, a protein expressed in neutrophil granulocytes, is a marker of intestinal inflammation. In accordance with our previous finding of increased gut permeability in MetS [20], zonulin and calprotectin levels were significantly elevated in stool but similar in serum of MetS patients compared to healthy controls. In contrast to animal and human studies that showed decreased faecal calprotectin levels by administration of a probiotic [61, 62], we could not find any influence of LcS supplementation on zonulin or calprotectin levels.

Bile acids have been proposed as potential modulators of gut permeability. In a mouse model high fat diet has been shown to decrease UDCA but increase DCA. In this model DCA increased gut permeability by a direct, non-inflammatory mechanism. [13, 14] *Ex vivo* data suggest that selected bile acids modulate intestinal permeability via rearrangement at the tight junction level. [63] In contrast to high fat diet models, genetically obese mice did not show increased gut permeability or bile acid pool hydrophobicity. [64] Furthermore, bile acids may directly impact on glucose metabolism. Dietary increase of bile acid pool size in a rat model resulted in a reduction of fat mass through an increase in energy expenditure. [65] A reduction of bile acid pool size increased body weight gain and worsened glucose intolerance induced by the high fat diet and led to a pronounced worsening of the changes in liver and adipose tissue. [66]

Human data are scarce. A biosynthetic bile acid precursor is increased in patients with MetS and T2DM, but this study did not investigate gut permeability. [67]. We could not find differences in the serum bile acid profiles between MetS patients and healthy controls. Bile acid

composition was not influenced by LcS treatment. Unfortunately we were not able to analyze bile acids in stool due to lack of material.

Our study has some limitations: Due to the small sample size individual differences might have outweighed the effects of LcS administration. Furthermore our healthy controls were significantly younger than our patient cohort. Age impacts on gut microbiota composition, but during adulthood the microbiota composition is relatively stable. [68] The *Bacteroidetes/Firmicutes* ratio decreases after infancy and rises again over the age of 70, but is reported to be stable in the age range of our controls and patients. [69]

This study suggests that investigating gut microbiota composition is challenging and interventions are difficult. Further studies are necessary to unravel the underlying mechanisms and find targeted therapeutic approaches for the complex interplay of gut microbiota and host metabolism.

Supporting Information

S1 CONSORT Checklist.

(DOCX)

S1 Study Protocol.

(PDF)

S1 Table. Barcoded primer sequences used in this study. Amplicons were sequenced from the Titanium A adaptor (CCATCTCATCCCTGCGTGTCTCCGAC), followed by a 4 bases key sequence (TCAG) and the 10 bases barcode. The reverse primer was used with the Titanium B adaptor (CCTATCCCCTGTGTGCCTTGGCAGTC), the key sequence and the target specific sequence but without barcode sequence (CCTATCCCCTGTGTGCCTTGGCAGTC TCAG ATTACCGCGGCTGCTGG).

(DOCX)

S2 Table. Abundance of gut microbiota concerning LcS supplementation. Median abundance is given for each group.

(DOCX)

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Author Contributions

Conceived and designed the experiments: VS HS. Performed the experiments: BL MT AH IK SL MB NJT. Analyzed the data: VS BL SL ST AH MB. Contributed reagents/materials/analysis tools: TS ST IK. Wrote the paper: VS BL SL ST MB AH MT TS GF PF CH IK PS ML TRP NJT HS.

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