Probiotics to Adolescents With Obesity: Effects on Inflammation and Metabolic Syndrome

*Rikke Juul Gøbel, †Nadja Larsen, †Mogens Jakobsen, *Christian Mølgaard, and *Kim Fleischer Michaelsen

ABSTRACT

Objectives: The connections between gut microbiota, energy homeostasis, and inflammation and its role in the pathogenesis of obesity-related disorders are increasingly recognized. We aimed to investigate the effect of the probiotic strain *Lactobacillus salivarius* Ls-33 on a series of biomarkers related to inflammation and the metabolic syndrome (MS) in adolescents with obesity.

Methods: The study was a double-blind placebo-controlled trial including 50 adolescents with obesity randomized to Ls-33 (10^{10} CFU) or placebo daily for 12 weeks.

Results: The average body mass index-for-age *z*-score was 2.6 ± 0.5. There were no differences in biomarkers of inflammation and parameters related to the MS at baseline between the probiotic and placebo groups. Furthermore, there were no differences in changes from baseline to 12-week intervention with regard to any anthropometric measures, blood pressure (systolic and diastolic), fasting glucose and insulin, homeostasis model assessment of insulin resistance, C-peptide, cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglyceride, free fatty acids, C-reactive protein, interleukin-6, tumor necrosis factor alpha, or fecal calprotectin, despite the increased values of biomarkers of inflammation and of several parameters related to the MS at baseline when compared with normal-weight adolescents. The levels of *L salivarius* in fecal samples from the probiotic group in the present study were comparable with the levels reported for the other probiotic lactobacilli and bifidobacteria using quantitative polymerase chain reaction.

Conclusions: It was not possible to detect any beneficial effect of the probiotic intervention with Ls-33 on inflammatory markers or parameters related to the MS in adolescents with obesity being in a state of low-grade systemic inflammation.

Key Words: adolescents, inflammation, metabolic syndrome, obesity, probiotic intervention

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Evidence from animal studies indicates that the composition of the gut microbiota is involved in extraintestinal disorders such as obesity (1–3). In a prospective study by Kalliomäki et al (4) it was reported that differences in the gut microbiota during the first year of life may precede the onset of obesity. The study established that the numbers of *Bifidobacterium* spp were higher and the numbers of *Staphylococcus aureus* were lower in children who remained at normal weight when compared with children who became overweight. Thus, a microbiota profile in favor of high numbers of bifidobacteria and low numbers of *S aureus* in infancy may protect against the development of obesity (5), identifying the gut microbiota as an additional contributing factor to the pathophysiology of obesity.

The number of human intervention studies on the effect of probiotics on body weight is limited. One recent study, however, describes the effect of a perinatal probiotic intervention on the development of overweight and obesity among children born by mothers receiving probiotics for 4 weeks before expected delivery and during breastfeeding/formula feeding until the age of 6 months (6). The child's body mass index (BMI) was measured from birth up to the age of 10 years, and the study concluded that modulation of the early gut microbiota with probiotics may modify the growth pattern of the child by restraining excessive weight gain during the first years of life.

There is, hitherto, a lack of studies looking into the specific effect of probiotics on the degree of the metabolic syndrome (MS) among adolescents with obesity. A few studies have, however, studied the effect of probiotics on specific elements of the MS in adults. One recent randomized clinical trial investigated the effects of *Lactobacillus acidophilus* NCFM on insulin sensitivity and the systemic inflammatory response in subjects with type 2 diabetes mellitus injected with *Escherichia coli* lipopolysaccharide (7). The inflammatory markers and the systemic inflammatory response were unaffected; however, the insulin sensitivity was preserved as a result of the probiotic intervention. Another recent study demonstrated that improved blood glucose control can be achieved among women in pregnancy and postpartum by dietary counseling combined with probiotics (*Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12) even in a normoglycemic population (8).

A series of animal studies have looked into the possible role of probiotics on aspects of the MS (9–11). In 1996, the effect of probiotics in pigs fed a high-cholesterol diet showed a reduction in serum cholesterol in pigs owing to probiotic ingestion (9). In relation to this, it has been shown that mice getting a probiotic mixture (*Saccharomyces boularii* and *Lactobacillus casei*

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From the *Department of Human Nutrition, and the †Department of Food Science, Faculty of Sciences, University of Copenhagen, Frederiksberg, Denmark.

Address correspondence and reprint requests to Rikke Juul Gøbel, Department of Human Nutrition, Faculty of Sciences, University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg, Denmark (e-mail: rg@dsr.life.ku.dk).

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NCDC19) significantly decreased serum cholesterol and low-density lipoprotein (LDL) cholesterol compared with control mice (11). Furthermore, the effect of the probiotic strain *L acidophilus* ATCC 43121 on cholesterol metabolism in hypercholesterolemia-induced rats has been investigated and supplementation with LAB did reduce total serum cholesterol with 25% and the sum of very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL) and LDL cholesterol with 42% (10). Studies on the effects of probiotics on human serum cholesterol are, however, conflicting and nonconclusive.

The connection between gut microbiota, energy homeostasis, and inflammation, and its role in the pathogenesis of obesity-related disorders are increasingly recognized. Despite this, studies investigating the effect of probiotics on obesity-related inflammation are few and mainly based on animal models. A recent study investigated the ability of yogurt in reducing gastrointestinal inflammation in a murine model and concluded that fermented milk could modulate the immune system and was able to attenuate the symptoms of acute inflammation by reducing inflammatory cytokines and increasing regulatory cytokine interleukin (IL)-10-producing cells, leading to desirable changes of the intestinal microbiota (12). There are, to our knowledge, no randomized clinical trials investigating the effect of probiotics on obesity-related inflammation among children or adolescents.

The aim of the present study was to investigate the effect of the probiotic strain, *Lactobacillus salivarius* Ls-33, on a series of biomarkers related to inflammation and the MS in adolescents with obesity.

METHODS

Study Design

The study was a double-blinded, randomized, placebo-controlled intervention study including 50 adolescents with obesity. Adolescents were between the age of 12 and 15 years at inclusion. Exclusion criteria were smoking, chronic diseases (with exception of mild forms of allergy), permanent use of medication, ingestion of antibiotics <1 month before first examination, and intake of yogurts and products containing probiotics for a minimum of 2 weeks before first examination. Recruitment was done via advertising in local newspapers and via health visitors of local schools. The recruitment period lasted from June 2009 to June 2010. Written consent from parents was obtained before inclusion. Adolescents were at inclusion randomized to receive a daily dose of either *L salivarius* Ls-33 ATCC SD5208 (10^{10} CFU) or placebo daily for 12 weeks. The random allocation sequence was generated with blocks of 8, and done using a computer model (*http://www.randomization.com*). Randomization and labeling of the intervention products were done by scientific personnel not involved in the study, and kept unavailable until data analysis was completed. The study was approved by the scientific ethical committees of the Capital Region of Denmark (H-B-2008-135). Adolescents of this study are equal to the obese group previously characterized (13).

Anthropometric and Blood Pressure Measures

Subjects came in for examination after overnight fast. The anthropometric measures included weight (Lindeltronic 8000, Digital Medical Scale, Copenhagen, Denmark) and height (Heightronic 235, QuickMedical Issaquah, WA). BMI-for-age *z* scores and height-for-age *z* scores were calculated using the WHO AnthroPlus-programme (*http://www.who.int/childgrowth/software/en*). Waist and hip circumference was measured using a flexible measuring tape (Meterex, Langenfeld, Germany). Waist circumference was measured at the midpoint between the lower ribs and the iliac crest. Hip circumference was measured horizontal at the largest circumference of the hip. Triceps and subscapularis skin folds were measured using standard methods (Harpenden Skinfold Calibre, Chasmors Ltd, London, UK) and body composition was measured using the multifrequency Quadscan 4000 (Bodystat, Douglas, UK). Blood pressure was measured at the brachial artery (upper arm) after 10 minutes of supine rest (A&D Medical, Tokyo, Japan).

Pubertal Stage

At time of inclusion the participants filled out a self-reported questionnaire on the stage of puberty according to the Tanner pubertal assessment questionnaire, based on breast developmental stage for girls (13) and pubic hair development stage for boys (14).

Blood Collection

Venous blood samples were drawn after a minimum of 9 hours of fasting, for measures of glucose, insulin, C-peptide, lipids, high sensitive CRP, IL-6, and tumor necrosis factor-alpha (TNF-α).

Glucose, Insulin, and C-peptide

Fasting plasma glucose was analyzed using the ABX Pentra Glucose HK CP kit on the Pentra 400 analyzers (Montpellier, France). The intra- and interassay variations were 0.7% and 2.5%, respectively. Fasting insulin were analyzed using the IMMULITE 1000 analyzers (Siemens, Los Angeles, CA) with intra-and interassay variation of 2.7% and 7.4%, respectively. C-peptides were analyzed using the IMMULITE 1000 analyzers having an intra- and interassay variation of 5.4% and 8.0%, respectively. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as glucose (mmol/L) × insulin (pmol/L)/135.

Blood Lipids

Serum triglyceride (TG), high-density lipoprotein (HDL), LDL, total cholesterol, and free fatty acids (FFAs) were analyzed using the specific ABX Penta kits on Pentra 400 analyzers. The intra- and interassay variations were 2.6% and 3.2%, respectively, for TG and 1.2% and 4.0% for HDL. The intra- and interassay variations were 1.3% and 2.7%, respectively, for LDL, 0.9% and 1.6% for total cholesterol, and 1.7% and 5.1% for FFA.

High-sensitivity C-reactive Protein (CRP)

High-sensitivity serum CRP was analyzed using the specific high-sensitivity CRP CP on Pentra 400 analyzers having a detection limit of 0.10 mg/L. The intra- and interassay variations were 3.6% and 8.1%, respectively.

IL-6 and TNF-α

Concentrations of IL-6 and TNF-α in plasma were determined by enzyme-linked immunosorbent assay (ELISA) and preparation of the assay was done as described by the manufacturer (PhiCal Test, Oslo, Norway).

Fecal Calprotectin

Stool samples were collected, kept in iceboxes, and brought to the laboratory within 24 hours where they were stored at −80°C

until analysis. Fecal calprotectin (FC) was analyzed by an ELISA, and preparation of the assay was done as described by the manufacturer (PhiCal Test). The detection limit of FC was 19.5 mg/kg.

Probiotic Cultures

Capsules with the freeze-dried probiotic strains *L salivarius* Ls-33 ATCC SD5208 and placebo were provided by Danisco Inc, and were undergoing continuous quality control with cell counts for the duration of the trial (Dansico Inc). There was no reduction in viability observed in the capsules for the duration of the trial. Capsules were stored at 5°C, and filler consisted of cellulose, silicondioxide, and rice-maltodextrin. The placebo consisted of filler material only. Probiotic cultures were ingested as 1 capsule containing the daily dosage of 10^{10} CFU during a period of 12 weeks.

Detection of *L salivarius* by Real-time Quantitative Polymerase Chain Reaction

Chromosomal DNA from fecal bacteria was extracted using the QIAamp DNA Stool Mini kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions with slight modifications (15). Species-specific quantitative polymerase chain reaction (qPCR) detection of *L salivarius* was performed with the use of the fluorogenic 5′ nuclease PCR assay and the 7500 fast real-time PCR System (Applied Biosystems, Foster City, CA). The forward primer For-Sal-3 (5′-GTCGTAACAAGGTAGCCG-TAGGA-3′), reverse primer Rev-Sal-1 (5′-TAAACAAAGTATTC-GATAAATGTACAGGTT-3′) and the minor groove binder (MGB) probe SalivarISRDes-4 (5′-CGGCTGGATCACC-3′) targeted 16S-23 intergenic spacer region (16). The assay was optimized with respect to primer/probe concentrations and annealing temperature. The total volume of PCR reaction mixture was 15 μL containing 1 ng of template DNA and 200 nmol/L of each primer and probe. The assays were conducted with the use of Universal TaqMan FAST Mastermix kit (Applied Biosystems). The amplification program consisted of AmpliTaq activation at 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and annealing at 59°C for 30 seconds. The standard strain was *L salivarius* ATCC11741. Bacteria in fecal samples were quantified in triplicates and expressed as mean quantity of bacteria per gram feces.

Statistical Analysis

The values of blood pressure (systolic and diastolic), TG, CRP, and TNF-α were log transformed to obtain a normal distribution; however, values were kept as non–log transformed values for comparison (Tables 1 and 2). Group differences between the intervention and the placebo group were analyzed with regard to age, sex, and pubertal stage (Table 3). Test for difference in age was calculated using independent samples *t* test. For sex a chi-square test was used and multinominal logistic regression adjusted for age was used for pubertal stage. Anthropometric measures, body composition, and blood pressure were analyzed by general linear model (GLM) multivariate analysis. Tests of differences with regards to BMI-for-age *z*-scores and height-for-age *z*-scores were unadjusted, whereas values of weight, height, waist, and hip circumference, waist/hip ratio, sum of skin folds, fat percentage, and blood pressure were adjusted for sex, age, and pubertal stage. Values of waist circumference were also adjusted for height (Table 1). Additionally, group differences in fasting levels of glucose and insulin, HOMA-IR, C-peptide, blood lipids, CRP, FC, IL-6, and TNF-α were analyzed (Table 2). Analyses were done by multivariate GLM analysis and adjusted for sex, age, and pubertal stage. Statistical analysis were done using SPSS version 18.0 (SPSS Inc, Chicago, IL). The level of significance considered was $P < 0.05$.

RESULTS

A total of 50 adolescents completed the study; 23 participants were randomized into the placebo group and 27 into the Ls-33 group. Owing to the block randomization (8 adolescents per block) and dropouts, the final number in each group ended out being

TABLE 1. Anthropometric characteristics of the adolescents in the study at baseline and after 12 weeks of probiotic intervention (N = 50)*

	Placebo, N = 23			Ls-33, N = 27			
	Baseline	After intervention	Diff†	Baseline	After intervention	Diff†	P‡
Weight, kg	84.5 ± 13.3	86.5 ± 13.4	2.0***	79.9 ± 15.4	81.5 ± 15.9	1.6***	0.60
Height, cm	167.3 ± 11.4	168.4 ± 11.6	1.1**	163.6 ± 9.0	164.8 ± 8.7	1.2**	0.83
BMI-for-age *z*-score	2.6 ± 0.4	2.6 ± 0.4	0.009ns	2.6 ± 0.5	2.6 ± 0.5	0.002ns	0.86
Height-for-age *z*-score	1.2 ± 1.2	1.2 ± 1.2	0.01ns	1.1 ± 1.0	1.1 ± 1.0	0.03ns	0.60
Waist, cm	103.6 ± 6.0	103.3 ± 6.6	−0.3ns	100.8 ± 9.1	101.4 ± 10.5	0.6ns	0.28
Hip, cm	106.9 ± 5.6	108.5 ± 6.2	1.6ns	105.1 ± 9.5	105.1 ± 9.4	−0.09ns	0.091
Waist/hip	1.0 ± 0.03	1.0 ± 0.05	−0.02ns	1.0 ± 0.05	1.0 ± 0.06	0.05ns	0.059
Skin folds, mm	68.5 ± 18.6	75.3 ± 18.2	6.8ns	67.8 ± 22.6	71.0 ± 21.4	3.2ns	0.56
Body fat, %	34.0 ± 4.5	33.9 ± 4.2	−0.1ns	33.2 ± 3.2	33.2 ± 4.6	0.02ns	0.75
BP sys, mmHg	115.0 ± 10.4	114.0 ± 8.3	−1.0ns	112.8 ± 7.5	112.0 ± 9.7	−0.8ns	0.83§
BP dia, mmHg	68.4 ± 5.1	67.1 ± 5.7	−1.3ns	69.6 ± 4.8	68.2 ± 7.3	−1.4ns	0.92§

BMI = body mass index; BP dia = blood pressure diastolic; BP sys = blood pressure systolic; diff = difference; nsNonsignificant difference between baseline and after intervention, $P > 0.05$.

* Values are expressed as mean ± standard deviation.

† Change from baseline to after intervention.

‡ *P* value describes differences between the changes of the intervention groups. *P* values of change from baseline to after intervention in BMI-for-age *z*-score and height-for-age *z*-score are calculated using the general linear model adjusted for baseline values. *P* values of change from baseline to after intervention in weight, height, waist, and hip circumference, sum of skin folds, body fat percentage, and BP are calculated by a general linear model analysis adjusted for baseline values, sex, age, and pubertal stage. The *P* value of waist circumference is additionally adjusted for height.

§ Values are log transformed before statistical tests to obtain a normally distribution.

** Significant difference between baseline and after intervention, $P < 0.01$.

*** Significant difference between baseline and after intervention, $P < 0.001$.

TABLE 2. Blood lipid profile and inflammatory markers before and after 12 weeks of probiotic intervention (N = 50)[†]

	Placebo, N = 23			Ls-33, N = 27			
	Baseline	After intervention	Diff[‡]	Baseline	After intervention	Diff[‡]	P[§]
Glucose, mmol/L	5.27 ± 0.30	5.19 ± 0.50	−0.08[ns]	5.18 ± 0.76	5.00 ± 0.36	−0.18[ns]	0.052
Insulin, pmol/L	109.00 ± 40.39	91.00 ± 31.03	−18.00[***]	104.68 ± 62.17	98.14 ± 57.92	−6.54[ns]	0.25
HOMA-IR[‖]	4.25 ± 1.60	3.51 ± 1.27	−0.74[*]	4.02 ± 2.39	3.63 ± 2.12	−0.39[ns]	0.51
C-peptide, pmol/L	920.30 ± 230.01	840.69 ± 184.31	−79.61[**]	857.63 ± 292.78	853.52 ± 266.80	−4.11[ns]	0.33
Cholesterol, mmol/L	4.21 ± 0.66	4.12 ± 0.56	−0.088[ns]	4.36 ± 0.72	4.15 ± 0.71	−0.21[ns]	0.87
HDL, mmol/L	1.09 ± 0.21	1.06 ± 0.22	−0.032[ns]	1.08 ± 0.21	1.05 ± 0.19	−0.028[ns]	0.77
LDL, mmol/L	2.50 ± 0.54	2.45 ± 0.44	−0.050[ns]	2.70 ± 0.60	2.54 ± 0.60	−0.16[ns]	0.97
TG, mmol/L	1.22 ± 0.66	1.16 ± 0.65	−0.068[ns]	1.10 ± 0.81	1.04 ± 0.74	−0.061[ns]	0.84[¶]
FFA, μmol/L	389.26 ± 117.85	475.78 ± 176.52	86.52[*]	339.33 ± 89.97	414.22 ± 132.48	14.89[ns]	0.24
CRP, mg/L	2.45 ± 2.69	3.48 ± 5.02	1.03[ns]	2.05 ± 2.44	1.78 ± 2.34	−0.26[ns]	0.33[¶]
IL-6, pg/mL	1.41 ± 0.89	1.34 ± 0.80	−0.074[ns]	1.46 ± 1.09	1.51 ± 0.75	0.052[ns]	0.53
TNF-α, pg/mL	3.66 ± 5.21	3.78 ± 5.40	0.11[ns]	2.43 ± 1.60	2.46 ± 1.52	0.020[ns]	0.74[¶]
FC, mg/kg	29.39 ± 15.53	25.24 ± 14.74	−4.15[ns]	37.13 ± 48.48	30.48 ± 21.46	−6.65[ns]	0.25

CRP = C-reactive protein; FC = fecal calprotectin; FFA = free fatty acid; HDL = high-density lipoprotein cholesterol; IL-6 = interleukin-6; LDL = low-density lipoprotein; HOMA-IR = homeostasis model assessment of insulin resistance; [ns]Nonsignificant difference between baseline and after intervention, $P > 0.05$; TG = triglyceride; TNF-α = tumor necrosis factor-alpha.

[†] Values are expressed as mean ± standard deviation.

[‡] Change from baseline to after intervention.

[§] P value describes differences in change from baseline to after intervention in the intervention groups. P values are calculated using the general linear model adjusted for baseline values, sex, age, and pubertal stage.

[‖] HOMA-IR = glucose (mmol/L) × insulin (pmol/L)/135.

[¶] Values are log transformed before statistical tests to obtain a normal distribution.

[*] Significant difference between baseline and after intervention, $P < 0.05$.

[**] Significant difference between baseline and after intervention, $P < 0.01$.

[***] Significant difference between baseline and after intervention, $P < 0.001$.

uneven (Fig. 1). There were no differences in age, sex, or pubertal stage between the groups at baseline (Table 3). Additionally there were no differences in weight, height, BMI-for-age z-score, height-for-age z-score, waist- and hip circumference, waist/hip ratio, sum of skin folds (triceps plus subscapularis), body fat percentage, or blood pressure (systolic and diastolic) between the groups at baseline (Table 1). The average BMI-for-age z-score was 2.6 ± 0.5. Fourteen percent of the adolescents fulfilled the criteria for MS according to the International Diabetes Federation definition (17). There were no differences between the groups at baseline in fasting glucose, insulin, HOMA-IR, C-peptide, variables included in the blood lipid profile (total cholesterol, HDL, LDL, TG, and FFA), or inflammatory markers (CRP, IL-6, TNF-α, and FC) (Table 2).

The weight and height of the adolescents increased significantly in both groups from baseline to after intervention, but there was no difference in the size of the change between the groups. The BMI-for-age z-score did not change significantly in either of the groups from before to after intervention.

Concentrations of fasting insulin, HOMA-IR, and C-peptide did decrease significantly from baseline to after intervention in the placebo group, but the change was not significantly different when compared with the Ls-33 intervention group. The concentration of FFA did increase significantly from baseline to after intervention in the placebo group, but again the change was not significantly different when compared with the Ls-33 group. The difference in fasting glucose from baseline to after intervention showed a tendency ($P = 0.052$) to a larger decrease in the Ls-33

TABLE 3. Baseline characteristics of the adolescents in the study (N = 50)[*]

	Placebo, N = 23	Ls-33, N = 27	P[†]
Age, y	13.4 ± 1.1	12.9 ± 1.0	0.55[‡]
Sex			
Female	12	16	
Male	11	11	0.40[§]
Pubertal stage			
Girls, Tanner (1:2:3:4:5)	(0:1:7:2:2)	(0:2:7:4:3)	
Boys, Tanner (1:2:3:4:5)	(0:4:3:1:3)	(1:5:3:1:1)	0.67[‖]

[*] Ages are expressed as mean ± standard deviation. Sexes are expressed as total numbers in the group, and category values of pubertal status are expressed as total numbers in each Tanner stage.

[†] P value describes differences between the placebo and Ls-33 groups.

[‡] t test.

[§] Chi-square test.

[‖] Multinominal logistic regression analysis adjusted for age.

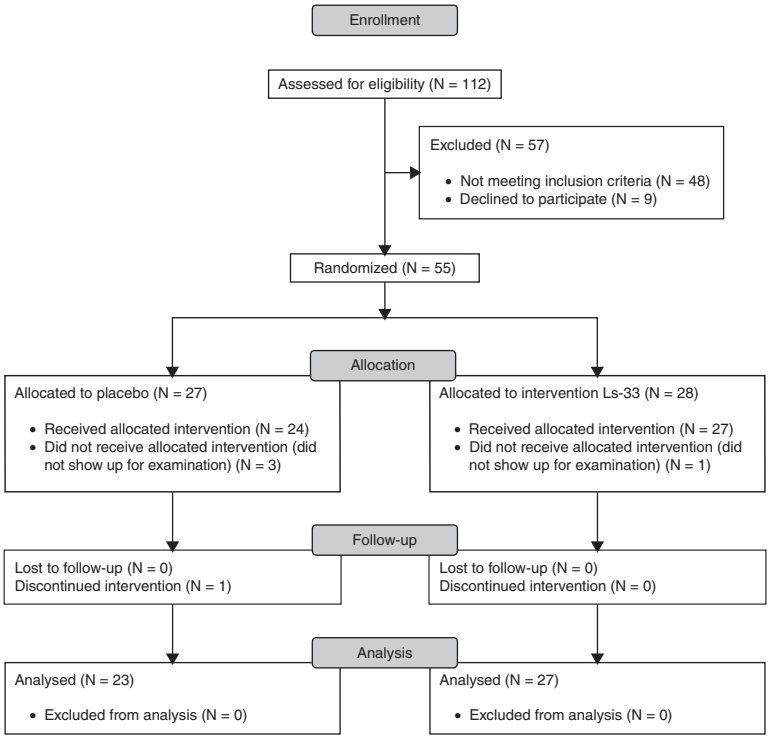

FIGURE 1. Flowchart according to the Consolidated Standards of Reporting Trials.

group when compared with the placebo group. There were no differences in change from baseline to after intervention between the groups, adjusted for baseline values, for any of the inflammatory markers.

Compliance

Compliance based on calculation of remaining capsules and self-reported diaries was in average 91.3%, (placebo 89.0% and Ls-33 93.5%). The adolescent with the lowest compliance ingested 60% of the capsules, whereas all of the remaining participants had compliance >70%. There was no difference in compliance between the 2 groups ($P = 0.94$).

Detection of *L salivarius* in Fecal Samples

L salivarius was detected in 24 of 27 subjects (89%) of the probiotic group after intake of Ls-33 at the mean level of 5.55 (± 0.95) $\log_{10}$ cells per gram stool (mean $\pm$ SD [standard deviation]). Other fecal samples from the probiotic group and placebo group were negative for *L salivarius*.

DISCUSSION

We found no effects of the intervention with the probiotic strain Ls-33 on either the inflammatory markers or the markers of MS. Adolescents included in the present study have previously been described and characterized (13), clearly showing that selected markers of inflammation and several parameters included in the definition of MS were substantially increased when compared with normal-weight adolescents. When using the International Diabetes Federation definition of MS among the adolescents in the present study the prevalence of the MS was 7 of the 50, corresponding to 14% (17). Another 19 adolescents did meet the obesity criteria of waist circumference >90th percentile plus 1 other criteria in the definition of the MS. Thus, these otherwise healthy obese Danish adolescents are in a stage of chronic low-grade inflammation having increased risk of a series of lifestyle-related diseases. The higher blood pressure and an unfavorable blood lipid profile, compared with normal-weight adolescents, together with the synergistic effects of other components of the MS, are additionally believed to increased their risk of cardiovascular diseases. On this background the included adolescents are believed to be the most suitable group for investigating the effect of probiotics on the selected biomarkers of inflammation and parameters related to the MS.

We chose to study the effects of the probiotic strain *L salivarius* Ls-33 on adolescents with obesity with subclinical inflammation and elevated risk factors for MS because in vitro and murine studies had suggested immunomodulatory and anti-inflammatory properties (18–21). One of these studies showed a protective effect of trinitrobenzene sulfonic acid–induced colitis in mice (18). Our study is the first to examine these effects in humans.

The study has several strengths, including the high compliance of >90%. The dosage and length of intervention were estimated as appropriated based on the existing literature of probiotic intervention trials within related research fields and strains. The selected dosage of 10^{10} CFU/day is in the upper end of the range when compared with studies that have reported probiotic effects (22).

There could be several reasons why we did not find an effect of the intervention. One possible explanation could be that results from in vitro and animal studies could not be translated to humans for this strain, leading to the overall discussion of cell and animal studies as effective models for humans. Second, the power of the study could be discussed. As no similar studies have been conducted within this area, an estimation of the variation in outcome parameters and relevant clinical effect of probiotics on obesity-related inflammation was done. With the included number of

50 obese children, which we aimed at, we estimated that we were able to detect a difference of 0.5 standard deviation in the primary outcome of CRP. As previously mentioned, it is our belief that the selected group of adolescents was not the reason for not being able to detect an effect of the intervention. Third, the compliance of the study was generally high (91.3%), based on calculation of remaining capsules and self-reported diaries. Besides, *L salivarius*, detected in 89% of fecal samples after probiotic intake, most probably represented the ingested strain, confirming satisfactory compliance of the volunteers and suggesting Ls-33 survival of the passage through the gastrointestinal tract. The levels of *L salivarius* in fecal samples from the probiotic group in the present study were comparable with the levels reported for the other probiotic lactobacilli and bifidobacteria using qPCR (15). Additionally, other probiotic strains of *L salivarius* have been recovered from child fecal samples after intervention (23,24).

Participation in a clinical trial with focus on body weight could potential have a positive effect on the lifestyle-related behavior of the adolescents. We observed a decrease in fasting insulin, HOMA-IR, and C-peptide in the placebo group during the 12-week duration of the trial; however, we cannot explain why the changes are only observed within the placebo group. It is well known that transient changes in insulin levels are known to be related to the normal physiological development during puberty (25–28). On the contrary, the present results showed a tendency to a decreased fasting glucose in the Ls-33 group compared to the placebo group when adjusted for baseline values ($P = 0.052$). A possible explanation may be that the results represent a random finding owing to the large number of parameters included.

In conclusion, it was not possible to detect any effects on either inflammatory markers or parameters related to the MS as a result of the probiotic intervention with *L salivarius* Ls-33 in adolescents with obesity being in a state of low-grade systemic inflammation; however, as effects of probiotics are often specific we cannot exclude that other strains will have beneficial effect in a populations with high levels of risk factors for later disease as in the group we examined with adolescents with obesity. More studies, especially RCTs, within this research area are needed.

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