Intake of *Lactobacillus reuteri* Improves Incretin and Insulin Secretion in Glucose-Tolerant Humans: A Proof of Concept

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A slide set summarizing this article is available online.

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OBJECTIVE

Ingestion of probiotics can modify gut microbiota and alter insulin resistance and diabetes development in rodents. We hypothesized that daily intake of *Lactobacillus reuteri* increases insulin sensitivity by changing cytokine release and insulin secretion via modulation of the release of glucagon-like peptides (GLP)-1 and -2.

RESEARCH DESIGN AND METHODS

A prospective, double-blind, randomized trial was performed in 21 glucose-tolerant humans (11 lean: age 49 ± 7 years, BMI 23.6 ± 1.7 kg/m^2; 10 obese: age 51 ± 7 years, BMI 35.5 ± 4.9 kg/m^2). Participants ingested 10^{10} b.i.d. *L. reuteri* SD5865 or placebo over 4 weeks. Oral glucose tolerance and isoglycemic glucose infusion tests were used to assess incretin effect and GLP-1 and GLP-2 secretion, and euglycemic-hyperinsulinemic clamps with [6,6-$^2H_2$]glucose were used to measure peripheral insulin sensitivity and endogenous glucose production. Muscle and hepatic lipid contents were assessed by ^{1}H-magnetic resonance spectroscopy, and immune status, cytokines, and endotoxin were measured with specific assays.

RESULTS

In glucose-tolerant volunteers, daily administration of *L. reuteri* SD5865 increased glucose-stimulated GLP-1 and GLP-2 release by 76% ($P < 0.01$) and 43% ($P < 0.01$), respectively, compared with placebo, along with 49% higher insulin ($P < 0.05$) and 55% higher C-peptide secretion ($P < 0.05$). However, the intervention did not alter peripheral and hepatic insulin sensitivity, body mass, ectopic fat content, or circulating cytokines.

CONCLUSIONS

Enrichment of gut microbiota with *L. reuteri* increases insulin secretion, possibly due to augmented incretin release, but does not directly affect insulin sensitivity or body fat distribution. This suggests that oral ingestion of one specific strain may serve as a novel therapeutic approach to improve glucose-dependent insulin release.

Type 2 diabetes results from decreased insulin sensitivity and inadequate insulin secretion, which associate with diminished incretin response and subclinical chronic inflammation and subsequent impaired glucose tolerance (1–4). These pathogenic factors, frequently accompanied by hypercaloric high-fat low-fiber diets, may be associated with alterations in gut microbiota, which also occur in obesity (5) and type 2 diabetes (6).

In animal models, modulation of intestinal microbiota may contribute to regulation of insulin resistance and postprandial glucose response (7). Mice treated with prebiotics exhibited reduced gut permeability and increased plasma glucagon-like peptide (GLP)-2 concentrations, which is cosecreted with GLP-1 and exerts an intestinotrophic effect. The improved gut barrier function further correlated with lower portal plasma lipopolysaccharide (LPS) (endotoxin) concentrations and lower systemic and hepatic inflammation (8).

Previous studies in humans and animals reported conflicting results on the effects of different *Lactobacillus* strains on body weight (9). In humans, ingestion of prebiotics has been shown to influence gut microbiota composition (10), perhaps alter intestinal permeability, and increase plasma GLP-1 concentrations and decrease postprandial glucose concentrations (11). Another recent study investigated the effects of allogenic fecal transplantation on glucose metabolism. Infusion of fecal preparations from lean donors transiently reduced insulin resistance in obese individuals with metabolic syndrome (12). Ingestion of *Lactobacillus acidophilus* NCFM for 4 weeks maintained insulin sensitivity without effect on the systemic inflammatory response in a metabolically heterogeneous group (13). Consumption of a yogurt containing *L. acidophilus* La5 and *Bifidobacterium lactis Bb12* over 6 weeks improved oxidative and glucose metabolism in patients with in type 2 diabetes (14). While the latter studies suggested a beneficial metabolic action on the host by *Lactobacilli*, even without modulating the microbiome (15), two double-blinded studies in obese adolescents failed to detect any beneficial effects of probiotics on the metabolic syndrome (16,17). At present, a randomized controlled intervention trial including analyses of tissue-specific effects and the possible underlying mechanisms is missing in metabolically well-phenotyped humans. Thus, this proof-of-principle study aimed at detailed examination of the effects of *Lactobacilli* on insulin secretion and action in humans. For this initial approach, healthy lean and obese individuals were recruited to test the effects without interference from glucose-lowering medication and variable degree of gut microbial dysbiosis (18,19).

We hypothesized that *L. reuteri* supplementation improves insulin sensitivity and increases insulin secretion by augmenting incretin release. Thus, we investigated the effect of ingested *L. reuteri* on incretin, tissue-specific insulin sensitivity, and β-cell function using isoglycemic and euglycemic clamps and measured fat distribution as well as markers of systemic inflammation in healthy lean and obese individuals. *L. reuteri* was selected for the intervention because of its well-studied safety and its strong probiotic activity compared with 46 other strains of *Lactobacillus spp.* (15,20).

RESEARCH DESIGN AND METHODS

Study Design

We performed a double-blind, 1:1 randomized, prospective, longitudinal pilot trial over 8 weeks. After a run-in phase, volunteers received BIO-*tract* controlled-release caplets with *L. reuteri* SD5865 at a dose of 2×10^{10} viable cells, encapsulated by Nutraceutix (Nutraceutix, Inc., Redmond, WA) and packaged identical to the placebo controls. Study participants started with a run-in phase of 4 weeks after the baseline visit during which they received placebo only. Then, they received capsules containing placebo or 10^{10} cells of *L. reuteri* to be ingested b.i.d. for 4 consecutive weeks.

Anthropometric measurements, oral glucose tolerance tests (OGTT), clamp studies, liver and muscle fat measurements, and collection of fecal samples were performed at the end of the run-in phase and at end of the trial. At each visit, participants underwent interviews regarding adverse effects, symptoms, or changes in quality of life. Compliance was assessed by counting capsules and by screening for *L. reuteri* in gut microbiota using quantitative real-time PCR. The study was performed according to the Declaration of Helsinki, approved by the local ethics committee, and registered at ClinicalTrials.gov. All participants provided written informed consent.

Participants

Lean (BMI 19–25 kg/m^2) and obese (30–45 kg/m^2) participants matched for age and sex were recruited at the German Diabetes Center. Inclusion criteria were age 40–65 years, nonsmoking, absence of gastrointestinal disease, and willingness to abstain from intake of other probiotic food and fermented milk products over the study period but otherwise stick to previous eating habits. Exclusion criteria were pregnancy, cancer, chronic diseases, treatment with antibiotics within 3 months prior to study inclusion and throughout study, competitive athletics, impaired glucose tolerance, and impaired fasting glucose.

Anthropometric Measurements

Body weight, height, waist circumference, and blood pressure were measured at each visit. Bioelectrical impedance analysis (Nutriguard-S; Data Input GmbH, Darmstadt, Germany) was performed in duplicates, and lean body and fat mass were calculated (21).

Gastric Emptying

Each subject underwent a [^{13}C]octanoic acid breath test to measure gastric emptying (22). In brief, the appearance of $^{13}CO_2$ in breath after oral administration of ^{13}C-octanoic acid applied with egg yolk was determined. Data were used to assess disturbed gastric emptying, as there is a reciprocal interplay of gut hormones and gastric motility.

OGTT and Isoglycemic Clamp Test

OGTT was performed after an overnight fast with 75 g glucose and 300 mL water (Dextrose O.G-T.; Roche Diagnostics, GmbH, Mannheim, Germany). β-Cell function was assessed from the insulinogenic index (23) and insulin sensitivity from the oral glucose insulin sensitivity index (OGIS) (24), which provides a measurement of insulin-mediated glucose clearance. The interplay between insulin sensitivity and secretion, which describes the β-cell adaptive response to changes of insulin resistance (25), was determined by the products OGIS $\times$ AUC_{CP} and OGIS $\times$ AUC_{INS}, where the AUCs are the area under the concentration curves of C-peptide and insulin, respectively. Adaptation and disposition index were originally developed for the intravenous glucose test but proved useful determinants of β-cell function also for OGTT (26). Further, the glucose AUC was used to measure differences in glucose tolerance upon intervention.

For measurement of the incretin effect, isoglycemic intravenous glucose infusion (20% in sterile water; Braun, Melsungen, Germany) was performed, after an overnight fast, over 3 h aiming at mimicking the blood glucose level measured during OGTT (27).

Euglycemic-Hyperinsulinemic Clamp Test

The clamp test was combined with isotopic dilution ([6,6-$^2H_2$]glucose) to assess both whole-body insulin sensitivity and hepatic insulin sensitivity. Constant insulin infusion (40 mU/m^2 body surface area i.v.; Insuman Rapid, Sanofi, Frankfurt, Germany) was given over 2 h, and blood glucose was adjusted to 80 mg/dL by variable 20% glucose infusion. Glucose infusion rate during the last 30 min during the clamp were used to calculate insulin sensitivity. Rates of endogenous glucose production (EGP) were determined from the tracer infusion rate of [6,6-$^2H_2$]glucose and its enrichment to hydrogens bound to carbon 6 divided by the mean percent enrichment of plasma [6,6-$^2H_2$]glucose. Steady-state equations were considered appropriate for calculation of basal EGP and insulin-suppressed EGP during the last 30 min of the clamp (28).

Indirect Calorimetry

For determination of in vivo whole-body energy expenditure and substrate oxidation from measuring oxygen consumption and carbon dioxide production, indirect calorimetry was performed, using Vmax Encore 29n (CareFusion, Höchberg, Germany) as previously described (28).

Determination of Ectopic Fat by ^{1}H-Magnetic Resonance Spectroscopy

Measurements were performed on a 3.0 Tesla magnet (X series; Achieva, Philips, Best, the Netherlands). Ectopic fat content from ^{1}H-magnetic resonance spectroscopy was quantified relative to internal water content and calculated as previously described (29). Data were analyzed using jMRUI software. Ectopic lipid content was assessed in eight lean (4 *L. reuteri* and 4 placebo) and seven obese (4 *L. reuteri* and 3 placebo) volunteers.

Analysis of Fecal Microbiota Composition and Content

For exact quantification of specific taxa, we performed quantitative real-time PCRs to detect total bacterial load (eubacteria), enterobacteria content, *L. spp*., and *L. reuteri*. In brief, total genomic DNA of the fecal samples was prepared within 24 h after collection and stored at −20°C until further analysis. DNA extraction was performed by using a BioRobot EZ1 machine (Qiagen, Hilden, Germany). Primers and probes used for qPCR were retrieved from the literature ("eubacteria" [30], "*Lac. spp.*" [31], "*L. reuteri*" [32]) or designed by Primer Express software (DNASTAR, Madison, WI) for enterobacteria content. Next-generation sequencing for analysis of fecal content was performed in cooperation with the University of Minnesota Genomics Center as described in detail in Supplementary Material 1.

Laboratory Measurements

Blood cell count, HbA_{1c}, hs-CRP, and blood glucose were measured by routine methods as previously described (28). Serum C-peptide, insulin, and plasma glucagon were measured by RIA (Millipore, St. Charles, MO). Cytokines and chemokines were assayed by the Flurokine MAP multiplex kit method (R&D Systems, Minneapolis, MN) and Luminex technology (Bio-Rad Bioplex, Nazareth, Belgium) (33). LPS was quantified using a chromogenic limulus amebocyte lysate QCL-1000 assay (Lonza, Ltd., Verviers, Belgium) according to the manufacturer's instructions. For validation of the assay, samples were spiked with known amounts of LPS and yielded a mean recovery rate of 90.4% (34). Production of reactive oxidative species was estimated from serum thiobarbituric acid–reactive substances (35). For incretin measurements, blood was collected into dipeptidyl peptidase-4 inhibitor–containing tubes (BD P700; BD Biosciences, Franklin Lakes, NJ). GLP-1 (7-36)amide and GLP-1 (9-36)amide and only fully processed GLP-2 of intestinal origin were measured as previously described (36,37). After deproteinization, determination of atom percent enrichment of ^{2}H was performed by gas chromatography–mass spectrometry as previously described (28).

Calculations and Statistics

Data are presented as mean ± SD or medians and interquartile range as appropriate. Variables with skewed distribution were log transformed before further analysis. To test differences between treatment arms taking the lean-obese status into account, we used two-way ANOVA. *P* values from two-sided tests ≤5% were considered to indicate significant differences. SAS for Windows version 9.2 (SAS Institute, Cary, NC) was used (Supplementary Material 2). For the individual hormones and metabolites, the total AUC was calculated using the trapezoidal rule. The incretin effect was assessed from comparing the total AUC values for insulin, C-peptide, glucagon, GLP-1, and GLP-2 during OGTT and with those obtained during the isoglycemic test. The ΔAUC was defined as the difference between the AUCs during OGTT and the isoglycemic test (Supplementary Material 2). Because of skewed distribution, the ΔAUCs were log transformed (natural logarithm). Differences of these ΔAUC values obtained before and after intervention were statistically analyzed.

RESULTS

Study Population

Thirty-nine persons were screened from October 2011 until August 2012. Thirteen persons did not pass the inclusion criteria. Of the remaining 21 participants, 5 lean received placebo and 6 lean received *L. reuteri*, whereas 5 obese received placebo and 5 obese received *L. reuteri* (Supplementary Fig. 1). Treatment adherence was 100% in all study groups (Table 1). None of the participants reported any adverse effects including gastrointestinal disorders.

Gut Microbiota Analyses

Fecal samples were obtained from all 21 participants before intervention and from all but 1 subject at the end of intervention. *L. reuteri* was not detectable before intervention in all but one lean subject of the placebo group. This subject was tested weakly positive (0.249 DNA copies) before intervention but lower after the intervention (0.041 DNA copies). All participants of the group treated with *L. reuteri* tested positive after intervention, while those of the placebo group were negative ($P < 0.0001$). In all groups, both treatments did not alter the total bacterial load, the enterobacteria, or the levels of total *Lactobacilli* (Supplementary Fig. 2).

Analysis of fecal microbiota composition by next-generation sequencing revealed that *L. reuteri* administration did not affect the overall composition of the fecal microbiota. No significant differences were observed when comparing temporal changes in relative abundance of bacterial taxa or operational taxonomic units between placebo-treated and *L. reuteri*–treated participants either when all participants were considered or when they were divided into lean and

Table 1—Baseline characteristics of study participants

	Combined	Lean	Obese
N (female/male)	11/10	6/5	5/5
Age (years)	50 ± 7	49 ± 7	51 ± 7
BMI (kg/m^2)	29.2 ± 7.0	**23.6 ± 1.7**	**35.5 ± 4.9***
Waist circumference (cm)	99.1 ± 17.7	**83.3 ± 11.5**	**111.9 ± 9.0***
Body fat (%)	34 ± 9	**28 ± 6**	**40 ± 9***
Lean body mass (kg)	56 ± 12	**50.7 ± 11.1**	**63.5 ± 9.5***
Fasting blood glucose (mg/dL)	78.3 ± 6.8	77 ± 9	79 ± 6
Fasting free fatty acids (μmol/L)	439 (370–631)	439 (246–522)	462 (386–678)
OGIS (mL/min/m^2)	528 ± 92	**569 ± 94**	**482 ± 67***
Adaptation index	0.808 (0.511–0.924)	0.615 (0.509–0.924)	0.830 (0.720–0.957)
Disposition index	3.09 (2.68–5.09)	2.76 (2.33–3.08)	4.77 (4.33–7.55)
Insulinogenic index	179 (123–396)	135 (94–409)	219 (147–395)
hs-CRP (mg/dL)	0.09 (0.05–0.29)	**0.05 (0.04–0.10)**	**0.30 (0.08–0.40)***
Systolic blood pressure (mmHg)	138 ± 21	133 ± 23	143 ± 16
Diastolic blood pressure (mmHg)	85 ± 11	**77 ± 9**	**93 ± 6***
Gastric emptying (t1/2)#	84 ± 34	90 ± 36	78 ± 32
Treatment adherence†	21/21	11/11	10/10

Mean ± SD and median (interquartile range) are given for normal and log-normal distributed data, respectively. Significant differences (*$P < 0.05$) as determined by unpaired *t* test between lean and obese persons are marked in bold. #Gastric emptying as measured by the ^{13}C-octanoic acid breath test is given as half-life (t1/2) in minutes. †Compliance was confirmed by tablet counting.

obese subgroups. α-Diversity analysis showed that the *L. reuteri* treatment did not modify the richness or evenness of the ecosystem. Principal coordinate analysis of β-diversity distance metrics did not show clustering due to the treatment (Supplementary Fig. 2). On an individual basis, these principal coordinate analyses suggest that, for most individuals, their fecal microbiota was relatively stable over time, as previously reported (38).

Anthropometry

The obese group had higher BMI ($P < 0.001$), body fat and lean body mass, hs-CRP, and diastolic blood pressure but lower OGIS in line with insulin resistance (Table 1). Body weight and resting energy expenditure of lean and obese participants differed ($P < 0.002$) but remained stable during the study and were not affected by the treatments (Supplementary Table 2) Waist-to-hip ratio and blood pressure also did not change (data not shown).

Ectopic Lipid Contents

Ingestion of *L. reuteri* did not affect hepatocellular lipids or intramyocellular lipids of m. soleus and m. tibialis anterior (Supplementary Table 2) in combined groups or in lean and obese subgroups. Hepatocellular lipids, but not intramyocellular lipids in both muscles, correlated with BMI before ($r = 0.72$, $P < 0.003$) and after ($r = 0.63$, $P < 0.014$).

Tissue-Specific Insulin Sensitivity and Substrate Oxidation

Whole-body insulin sensitivity was not affected by intervention. The *L. reuteri* and placebo groups showed no differences before or after intervention (Supplementary Fig. 3*A*). Steady-state conditions of glucose, insulin, C-peptide, and cortisol were comparable. Lean participants showed better whole-body insulin sensitivity compared with obese before ($P < 0.05$) and after ($P < 0.05$) intervention. In line with this, lipid oxidation rates during hyperinsulinemia were reduced by *L. reuteri* (94%) and placebo (102%) compared with baseline without differences between lean and obese. Oxidative glucose utilization and nonoxidative glucose utilization were increased during clamp but not affected by treatment (Supplementary Fig. 3*B*) and showed no differences in subgroup analysis for lean and obese.

L. reuteri did not affect EGP at baseline or during EGP suppression, being comparable between *L. reuteri* and placebo groups before and after intervention (Supplementary Fig. 3*C*) as well as in subgroup analysis of lean and obese.

Glucose Tolerance

Blood glucose levels during OGTT remained unchanged upon treatment (Supplementary Fig. 4*A*). Likewise, isoglycemic intravenous glucose infusion (Supplementary Fig. 4*B*) and the total glucose infusion rate showed no differences in the intervention or placebo group. Glucose infusion rate of lean and obese persons differed after (median 13.0 [interquartile range 6.9–29.6] vs. 37.5 [22.0–47.9], $P < 0.004$) but not before (23.2 [10.3–26.2] vs. 22.3 [19.3–51.3], $P = 0.3073$) intervention, while subgroup-analysis of lean and obese participants showed no treatment effect.

Insulin Secretion and β-Cell Function

After intervention ΔAUC insulin and C-peptide were increased in the *L. reuteri* ($P < 0.05$), but not the placebo group (Fig. 1*A* and *B*). The mean increase of the ΔAUC insulin concentrations was 49% (46% for lean and 51% obese; significant only for combined group) and for C-peptide 55% (39% for lean and 74% obese; significant only for combined group). Increased concentrations were seen for insulin and C-peptide at 20 and 60 min of oral glucose stimulation in the *L. reuteri* group at the end of the trial ($P < 0.05$) (Fig. 1*A* and *B*). Maximal response for insulin and C-peptide was increased at the end of the study in the intervention group ($P < 0.016$, $P < 0.018$, respectively). Additionally, adaptation, disposition, and insulinogenic indices were increased in the *L. reuteri* group ($P < 0.05$) upon treatment compared with placebo group (Supplementary Table 2).

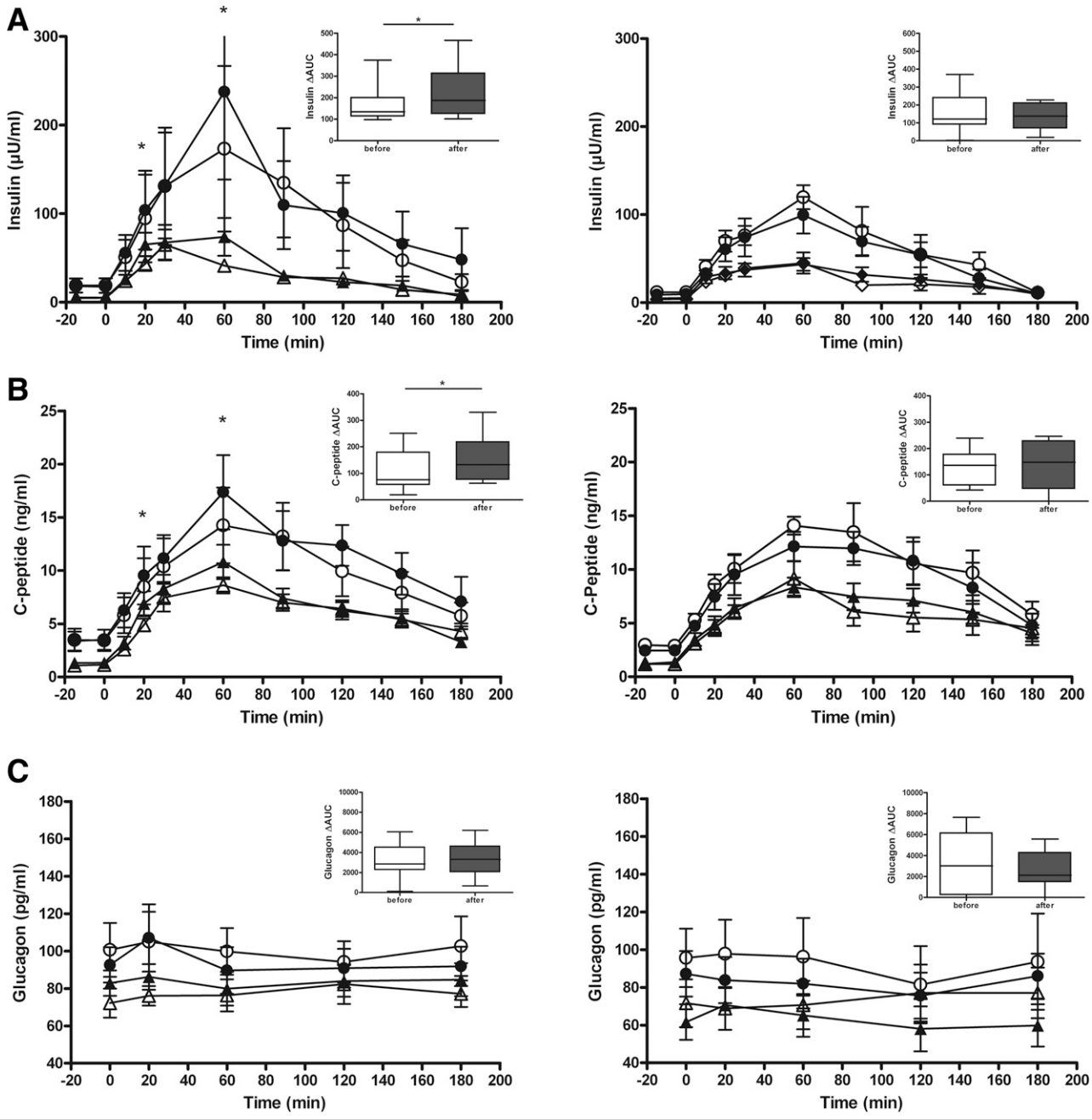

Figure 1—Insulin, C-peptide, and glucagon. Insulin, C-peptide, and glucagon concentrations during OGTT before (open symbols) and after (closed symbols) intervention of *L. reuteri* and placebo groups, respectively. Shown are data from lean (triangles) and obese (circles) persons as mean ± SD. Inserts show combined group (lean and obese) ΔAUC before vs. after intervention of *L. reuteri* and placebo groups. Shown are boxes and whiskers. Error bars represent the minimum and maximum value. *$P < 0.05$ vs. baseline for combined group.

Subgroup analysis showed differences upon treatment for the adaptation index ($P < 0.022$) and the disposition index ($P < 0.033$) only in the obese group.

Incretin Response and Incretin Effect

Administration of oral glucose leads to increased levels of GLP-1 and GLP-2 within a maximum at 30 min for GLP-1 and 60 min for GLP-2 (Fig. 2*A* and *B*).

Maximal responses for GLP-1 and GLP-2 were increased at the end of the study in the intervention group for lean participants ($P < 0.02$, $P < 0.04$, respectively) but not for obese participants.

Fasting plasma GLP-1 levels were different within the intervention group after treatment ($P < 0.02$, 0 min) and were by trend increased ($P = 0.052$, 0 min) compared with placebo. Glucose-stimulated GLP-1 levels were higher after treatment ($P < 0.05$) within the *L. reuteri* group (Fig. 2*A*) and compared with placebo ($P < 0.05$). Similarly, glucose-stimulated GLP-2 levels tended to increase after treatment ($P = 0.056$ and $P = 0.065$, respectively) within the *L. reuteri* group (Fig. 2*B*) and increased compared with placebo (20 min: $P < 0.05$, 60 min: $P < 0.02$).

Glucose-stimulated AUC of GLP-1 was increased in the *L. reuteri* group after

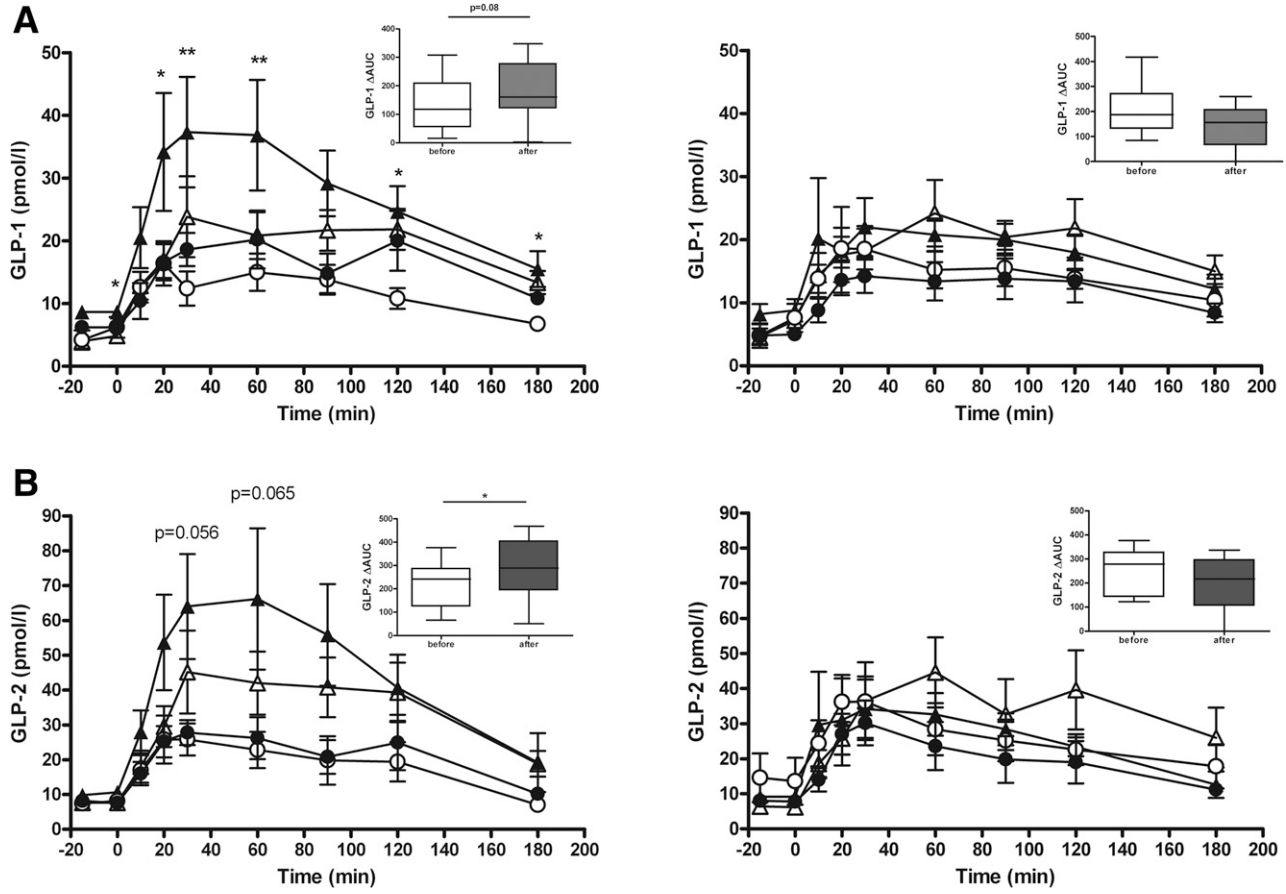

Figure 2—GLP-1 and GLP-2 concentrations during OGTT. GLP-1 and GLP-2 concentrations during OGTT before (open symbols) and after (closed symbols) intervention. Shown are lean (triangles) and obese (circles) persons as mean ± SEM. Inserts show combined group (lean and obese) ΔAUC before vs. after intervention of *L. reuteri* and placebo groups. Shown are boxes and whiskers. Error bars represent the minimum and maximum value. $*P < 0.05$, $**P < 0.01$ vs. baseline for combined group.

intervention ($P < 0.003$) and enlarged compared with the placebo group (Fig. 2*A*). Similarly, glucose-stimulated AUC of GLP-2 was increased in the intervention group compared with placebo ($P < 0.029$) (Fig. 2*B*).

ΔAUC of GLP-1 was increased at the end of the study compared with placebo ($P < 0.004$) and tended to increase within the *L. reuteri* group ($P = 0.087$) (Fig. 2*A*). *L. reuteri* increased ΔAUC of GLP-2 ($P < 0.02$) significantly different from placebo ($P < 0.004$) (Fig. 2*B*).

Concentrations of GLP-1 and GLP-2 during OGTT showed a similar pattern and correlated positive intraindividually before (mean $r = 0.69$; $P < 0.0001$) and after (mean $r = 0.73$; $P < 0.0001$) intervention. Additionally, during OGTT GLP-1 and GLP-2 correlated intraindividually with insulin and C-peptide (all $P < 0.001$) but not during the isoglycemic test (data not shown). Likewise, AUCs of GLP-1 and GLP-2 correlated before ($r = 0.87$, $P < 0.0001$) and after ($r = 0.95$, $P < 0.0001$) intervention.

Endotoxin, Cytokines, and Oxidative Stress

Fasting serum endotoxin levels were higher in obese compared with lean participants before intervention ($P < 0.019$) but not afterward ($P = 0.169$) (Supplementary Fig. 5). Serum endotoxin levels correlated with BMI before ($r = 0.443$, $P < 0.045$) but not after ($r = 0.354$, $P = 0.115$) intervention. Before and after intervention, obese participants had higher fasting hs-CRP ($P < 0.004$ and $P < 0.002$) as well as higher interleukin (IL)-1ra, MCP-1, and tumor necrosis factor-α. IL-8 and MIP-1β were not different between groups and interventions. Ingestion of *L. reuteri* did not affect the ratios between analyzed pro- and anti-inflammatory immune mediators, including the tumor necrosis factor-α–to–IL-1ra ratio. Measurements of γ-interferon, IL-6, IL-1β, and IL-10 concentrations yielded >80% of values below the detection limit and were therefore not further analyzed. Finally, serum thiobarbituric acid–reactive substances, as a measure of reactive oxygen species differed neither between groups nor between interventions (Supplementary Table 3).

CONCLUSIONS

This study demonstrates that ingestion of *L. reuteri* modulates the secretion of insulin, C-peptide, and proglucagon-derived gut peptides in humans, whereas insulin sensitivity and parameters influencing insulin sensitivity such as body mass and ectopic fat content, as well as systemic inflammation or oxidative stress, remain unchanged. Stool samples from persons ingesting *L. reuteri* were positive tested; placebo-treated persons stayed negative, while no differences were detected in other bacterial

members of the fecal microbiota. In line with our results in humans, Poutahidis et al. (15) showed that ingestion of *L. reuteri* has an effect on the host physiology of mice, altering immunological and metabolic aspects without changing the gut microbial ecology.

GLP-2 primarily targets intestinal mucosa promoting growth and intestinal integrity (8). Both GLP-1 and GLP-2 are processed from proglucagon with equal efficiency and secreted by the intestinal L cells in parallel and in equimolar amounts (37). Indeed, GLP-1 and GLP-2 measured in our study were positively correlated. GLP-1 and GLP-2 correlate intraindividually with insulin and C-peptide concentrations during OGTT but not during the isoglycemic test. The association of GLP-2 concentrations with those of insulin probably is indirect and explained by the cosecretion of GLP-2 with the hormone GLP-1. After treatment with *L. reuteri*, the mean increase of GLP-2 ΔAUC during OGTT was 43% compared with baseline and this was similar to the 76% increase seen for GLP-1 ΔAUC. An involvement of both peptides, the intestinotrophic GLP-2 and insulinotrophic GLP-1, in effects of a prebiotic treatment was found previously in animal models (8) and even in germ-free mice (39). Our study complemented these findings, showing that the probiotic strain *L. reuteri* increases the glucose-stimulated release of GLP-1 and GLP-2 in glucose-tolerant human participants.

The increased GLP-1 concentrations can serve to explain the increased glucose-stimulated insulin secretion in the intervention-group. The mean ΔAUC insulin was increased by 49% (46% for lean, 51% obese). This was paralleled as expected by an increase of ΔAUC C-peptide by 55% (39% for lean, 74% obese) and also accompanied by altered β-cell function, determined by insulinogenic adaptation and disposition index. At first sight, the increased insulin secretion observed in association with increased GLP-1 and β-cell function suggests that *L. reuteri* ingestion would offer a potential means to apply this regimen in patients with type 2 diabetes. However, as the investigated participants were healthy, whether the increased incretin and insulin concentrations obtained here can be extrapolated to patients with type 2 diabetes remains unresolved and is of physiological relevance.

There was no impact on hepatic and peripheral insulin sensitivity, glucagon concentrations, or hepatocellular and myocellular lipid content by *L. reuteri* administration. The only other study of the probiotic effect on insulin sensitivity reported that consumption of *L. acidophilus* NCFM for 4 weeks in a heterogeneous study population of 54 males with type 2 diabetes or with impaired or normal glucose tolerance revealed stable insulin sensitivity compared with the placebo group (13). There was considerable variability among study participants, and it was not reported whether those with an increase of insulin sensitivity were diabetic or normoglycemic. Only in the latter case would there be a difference from our observations in glucose-tolerant participants.

The relationship between insulin sensitivity, insulin level, and glucose tolerance is to some extent mediated by free fatty acid (4). However, there were no alterations of free fatty acid concentrations due to intervention (Supplementary Fig. 6), whereas *L. reuteri* changed incretin concentrations and insulin secretion.

At least one study suggested beneficial effects of probiotics on liver disease such as nonalcoholic fatty liver disease and alcoholic liver cirrhosis in humans (40). In our study, the hepatocellular and myocellular lipid content of all participants remained unchanged, which may again be due to the fact that we studied healthy persons.

Our study in healthy lean and obese persons with normal glucose tolerance did not show alterations of blood glucose or HbA_{1c} levels. Interestingly, a recent prospective dietary analysis showed that greater intake of yogurt is associated with decreased risk of type 2 diabetes (41).

We also searched for additional metabolic effects of the ingestion of *L. reuteri* that involve improved incretin release. An increase of GLP-2 secretion most probably enhance the expression of tight junctions in the intestinal wall, with the consequence of decreased gut permeability and leakage of endotoxin (8). It has been suggested that low-grade metabolic inflammation driven by endotoxin translocation is the proposed mechanism by which the microbiota may contribute to systemic inflammation in obesity due to increased intestinal permeability (7,8). *Lactobacilli* may have the capability to improve intestinal integrity in rodents, which may diminish the LPS overflow from the gut to the circulation and thereby reduce the systemic concentrations of inflammatory markers (42). In our study, LPS and systemic cytokine concentrations remained stable.

Limitations of our study are the small-sized cohorts and the rather short intervention period. However, contrary to other studies in the field, where healthy control subjects and patients with metabolic disorders were analyzed together, we investigated healthy people without diabetes and extensively characterized them metabolically by validated methods including euglycemic-hyperinsulinemic clamp test with isotopic dilution to assess tissue-specific insulin sensitivity and substrate oxidation, different calculations of insulin sensitivity and β-cell function.

Although our study failed to detect physiological consequences of an increased incretin, insulin and C-peptide response in the *L. reuteri* group, these cannot be excluded. With a treatment duration of 4 weeks, our study was not designed to test for long-term effects and changes in body weight were not expected. The slightly increased incretin and insulin concentrations are within the physiological range of metabolic responses, which will not likely affect glucose tolerance of healthy humans. However, it remains to be tested whether patients with type 2 diabetes would respond differently than healthy volunteers.

In conclusion, administration of probiotic *L. reuteri* increased insulin secretion and incretin release in humans. This effect was not caused by a modulation of the fecal microbiota, suggesting a direct effect of the *Lactobacilli* on host physiology. The increase of the intestinotrophic gut peptide, GLP-2, which may contribute to intestinal integrity, was accompanied by stable concentrations of endotoxin and immune mediators. Therefore, administration of a specific bacterial strain might have clinical implications by improving incretin-mediated β-cell function in individuals with impaired glucose homeostasis and therefore warrants further studies on specific bacterial strains in type 2 diabetes.

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Author Contributions. M.-C.S., H.K., P.S., N.C.S., and M.R. designed the study. M.-C.S., B.N., and F.Z. performed the research. M.-C.S., B.N., P.N., V.B., F.Z., J.-H.H., G.P., B.H., J.J.H., C.M.K., L.B.B., I.M., J.W., and B.H. generated data. M.-C.S., K.S., and I.M. performed the statistical analysis. M.-C.S., H.K., N.C.S., and M.R. drafted the manuscript. All authors critically reviewed the manuscript. M.R. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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