

Effects of *Lactobacillus acidophilus* NCFM on insulin sensitivity and the systemic inflammatory response in human subjects

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According to animal studies, intake of probiotic bacteria may improve glucose homeostasis. We hypothesised that probiotic bacteria improve insulin sensitivity by attenuating systemic inflammation. Therefore, the effects of oral supplementation with the probiotic bacterium *Lactobacillus acidophilus* NCFM on insulin sensitivity and the inflammatory response were investigated in subjects with normal or impaired insulin sensitivity. In a double-blinded, randomised fashion, forty-five males with type 2 diabetes, impaired or normal glucose tolerance were enrolled and allocated to a 4-week treatment course with either *L. acidophilus* NCFM or placebo. *L. acidophilus* was detected in stool samples by denaturing gradient gel electrophoresis and real-time PCR. Separated by the 4-week intervention period, two hyperinsulinaemic–euglycaemic clamps were performed to estimate insulin sensitivity. Furthermore, the systemic inflammatory response was evaluated by subjecting the participants to *Escherichia coli* lipopolysaccharide injection (0.3 ng/kg) before and after the treatment course. *L. acidophilus* NCFM was detected in 75% of the faecal samples after treatment with the probiotic bacterium. Insulin sensitivity was preserved among volunteers in the *L. acidophilus* NCFM group, whereas it decreased in the placebo group. Both baseline inflammatory markers and the systemic inflammatory response were, however, unaffected by the intervention. In conclusion, intake of *L. acidophilus* NCFM for 4 weeks preserved insulin sensitivity compared with placebo, but did not affect the systemic inflammatory response.

Hyperinsulinaemic–euglycaemic clamp technique: Inflammation: Insulin sensitivity: Lipopolysaccharides: Probiotics

Recent studies implicate probiotic bacteria as putative anti-diabetic agents, since probiotic supplementation has been found to normalise glucose homeostasis in diabetic rats^(1–3). The underlying mechanisms are presently unknown, but since probiotics are increasingly incorporated in food, particularly dairy products, and consumed in significant quantities worldwide, such effects may have a huge impact on clinical practice, notably with regard to dietary recommendations to both healthy individuals at risk of type 2 diabetes as well as patients with type 2 diabetes. Probiotic bacteria may be defined as ‘living microorganisms, which, when administered in adequate amounts, confer a health benefit on the host’^(4,5). After surviving the passage of the gastrointestinal tract, probiotics may persist as part of the gut microbiota for a certain period after intake^(6,7). There is growing evidence that the intestinal microbiota exert effects outside the gut, for example, by regulating whole-body energy balance through mechanisms associated with breakdown and uptake of gut contents^(8,9). In addition, the intestinal microbiota may be crucial to the development and homeostasis of the immune system since

most immune cells in the body are conditioned in the gut and the gut microbiota interact closely with intestinal immune cells^(10–13). Processes such as energy harvest and immunity could therefore be affected by changes in the composition of the intestinal flora, induced by oral supplementation of probiotics. Furthermore, specific probiotic strains improve intestinal barrier function⁽¹⁴⁾, and may potentially diminish the translocation of micro-organisms and their derivatives, for example, lipopolysaccharide (LPS)⁽¹⁵⁾, from the gut to the systemic circulation, thereby reducing the concomitant release of pro-inflammatory cytokines via Toll-like receptor (TLR)-4 signalling⁽¹⁶⁾. So-called chronic low-grade inflammation with persistently elevated levels of circulating pro-inflammatory cytokines is considered a principal pathogenetic component of insulin resistance and type 2 diabetes^(17–19); we therefore speculated that probiotics may possess anti-diabetic properties, at least in part, due to immune-modulatory effects. *Lactobacillus acidophilus* NCFM is a probiotic strain that is widely applied in conventional foods such as dairy products and in dietary supplements.

Abbreviations: IGT, impaired glucose tolerance; IL-1ra, IL-1 receptor antagonist; LPS, lipopolysaccharide; *M/I*, estimate for insulin sensitivity; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test.

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L. acidophilus NCFM has been demonstrated to interact with cells of the immune system^(20,21) and increases intestinal integrity⁽²²⁾. The *L. acidophilus* NCFM strain has been investigated for its physiological, biochemical and fermentative properties *in vitro* and in animal studies, as reviewed by Sanders & Klaenhammer⁽²³⁾, and its genome has been fully sequenced⁽²⁴⁾. To our knowledge, well-controlled interventions on insulin-resistant or diabetic patients who consumed *L. acidophilus* NCFM have not been performed. The objective of the present study was therefore, in a randomised, double-blinded, placebo-controlled fashion, to test the hypothesis that oral supplementation with *L. acidophilus* NCFM improves insulin sensitivity and modulates systemic inflammation in human subjects. Hence, we investigated insulin sensitivity through hyperinsulinaemic–euglycaemic clamps⁽²⁵⁾ before and after a 4-week treatment course with oral supplementation of *L. acidophilus* NCFM or placebo. This was carried out in healthy and type 2 diabetic individuals, representing a wide continuum of insulin sensitivity. Furthermore, the effects of *L. acidophilus* NCFM on the systemic inflammatory response was evaluated by subjecting the participants to an intravenous injection of *Escherichia coli* LPS, an established and validated human *in vivo* model of systemic inflammation⁽²⁶⁾, before and after the treatment course.

Subjects and experimental methods

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethical Committee of Copenhagen and Frederiksberg Municipalities (KF 01-320695). Written informed consent was obtained from all subjects.

Subjects

After informed consent, forty-eight males, diagnosed with type 2 diabetes ($n = 24$) or as being non-diabetic ($n = 24$) by their general practitioner, were subjected to a thorough physical examination including a complete medical history, blood sampling for general medical screening and a fasting 2 h oral glucose tolerance test (OGTT). According to the OGTT, twenty subjects had type 2 diabetes, five had impaired glucose tolerance (IGT) and twenty-three had normal glucose tolerance (NGT) as classified by the WHO definitions of glucose tolerance⁽²⁷⁾. Of the twenty diabetic patients, a total of fourteen received medical treatment related to their condition: nine subjects were treated with metformin, three with sulfonylurea drugs, one with glitazones, and five with statins, either as monotherapy or in combination with each other. Volunteers suffering from inflammatory illnesses or being treated with insulin, steroids, non-steroidal anti-inflammatory drugs, anti-coagulants, angiotensin-converting enzyme inhibitors or angiotensin II antagonists were excluded.

Design

The study was conducted as a randomised, placebo-controlled, double-blinded trial. The volunteers were randomly assigned to intake of *L. acidophilus* NCFM ($n = 24$) or placebo ($n = 24$)

for a period of 4 weeks, ensuring that twelve subjects with NGT and twelve patients with diabetes as diagnosed by their general practitioner were included in each treatment group. The participants were instructed to abstain from fermented dairy products during treatment, but otherwise maintained their usual diet and lifestyle. Strenuous physical exercise was avoided for 48 h before the OGTT and the experimental days, while oral anti-diabetics and statins were withheld for 1 week and all other medications for 24 h. A hyperinsulinaemic–euglycaemic clamp and an investigation of the LPS-induced systemic inflammatory response, respectively, were performed on two consecutive days immediately preceding the intervention as well as on two consecutive days immediately after the treatment period. The clamps were always executed the day before the LPS injection.

Oral glucose tolerance test

Subjects drank 75 g glucose diluted in 500 ml water over 5 min. Plasma glucose was measured at baseline, and at 1 and 2 h after the glucose administration.

Dual X-ray absorptiometry scan

Fat-free body mass was estimated by dual X-ray absorptiometry scanning (Lunar Prodigy Advance; GE Medical Systems Lunar, Milwaukee, WI, USA) using Prodigy software (enCORE 2004, version 8.8; GE Lunar Corp., Madison, WI, USA).

Lactobacillus acidophilus NCFM and placebo

Capsules with freeze-dried *L. acidophilus* NCFM, American Type Culture Collection (ATCC) 700396 (1 g; about 10^{10} colony-forming units), or identical capsules containing placebo, a mixture of silicon dioxide and lactose, ratio 1:1, were provided by Danisco Inc. (Madison, WI, USA), and stored at 0–5°C before use. Bacterial viability was confirmed at the Department of Food Science, Faculty of Life Science, University of Copenhagen by plating serial dilutions of bacterial suspensions onto de Man–Rogosa–Sharpe agar (Merck, Darmstadt, Germany), followed by incubation in an anaerobic jar with AnaeroGen™ (Oxoid Ltd, Basingstoke, Hants, UK) at 37°C for 48 h.

Stool sampling and detection of Lactobacillus acidophilus

Stool samples were collected 1–2 d before the intervention was initiated and on the last day of intervention. After defecation, samples were kept at 5°C and brought to the laboratory within 24 h, where they were stored at –80°C until analysed. Total bacterial DNA was extracted from faecal samples using the QIAamp DNA Stool Mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. *L. acidophilus* NCFM chromosomal DNA was extracted using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, Munich, Germany). Bacterial DNA was amplified with the use of *Lactobacillus* group-specific primers Lac1F (AGCAGTAGGGAATCTTCCA) and Lac2R (GC clamp –ATTYCACCGCTACATG) under PCR conditions as described previously⁽²⁸⁾. PCR products were separated using the DCode System (Bio-Rad, Hercules, CA, USA)

according to the protocol for denaturing gradient gel electrophoresis⁽²⁹⁾. DNA fragments in faecal samples with an identical migration position as the PCR band obtained from *L. acidophilus* NCFM were assumed to represent *L. acidophilus*. This assumption was confirmed by sequencing of five random DNA fragments having the same running distances as *L. acidophilus* NCFM⁽³⁰⁾; identification was done using the BLAST tool at the National Centre for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov/BLAST>). *L. acidophilus* was further quantified by real-time PCR (quantitative PCR) with the use of species-specific primers: forward primer CTGCTGTTTCTTCAGCATCT and reverse primer TCAGTATTGATACCACGTGAAT. The primers were constructed from *Lactobacillus* cpn60 gene sequences (Chaperonin Database; <http://cpndb.cbr.nrc.ca/>) by sequence alignment (ClustalW; <http://www.ebi.ac.uk/Tools/clustalw2/index.html>) followed by identification of specific primer sites ('Oligo' Primer Analysis Software version 6.71; Molecular Biology Insights, Inc., Cascade, CO, USA). Specificity of the primers was evaluated *in silico* (BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Quantification of *L. acidophilus* was carried out using the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The reaction mixture (20 µl) was composed of 0.5 µM of each primer, 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems) and 4 µl template DNA isolated from faecal samples. The amplification program consisted of one cycle of 95°C for 10 min, followed by forty cycles of 95°C for 15 s, and 60°C for 1 min. Standard curves were constructed using 10-fold serial dilutions of chromosomal DNA, isolated from *L. acidophilus* NCFM. Cell numbers of *L. acidophilus* in faecal samples were calculated from the averaged threshold cycle values (Ct) and expressed as log₁₀ colony-forming units per g stool.

Hyperinsulinaemic–euglycaemic clamp

After an overnight fast, subjects reported to our laboratory, where they were bed-rested. An intravenous catheter for administration of insulin, glucose and electrolytes was placed in an antecubital vein. A retrograde catheter was inserted into a dorsal vein in the contralateral hand; this hand was wrapped in a heating blanket to obtain arterialised venous blood for measurements as specified below.

The duration of the clamp was 180 min. Insulin (100 IU/ml; Actrapid, Novo Nordisk Insulin, Bagsværd, Denmark) was infused continuously at a rate of 0.120 IU/min per m². Plasma glucose and K was measured at 5 min intervals during hour 1 and at 10 min intervals during the hours 2 and 3; plasma glucose levels were targeted at 5.0 ± 0.2 mmol/l by adjusting the infusion rates of glucose (200 g/l) on a computer-controlled infusion pump. *M*, the whole-body metabolic rate of glucose, was calculated from the glucose infusion rate in the interval between 120 and 180 min⁽³¹⁾ according to the equations of DeFronzo *et al.*⁽²⁵⁾. The ratio of *M* (in mg/min per kg fat-free body mass) to the mean plasma insulin concentrations (in nmol/l) (*M/I*) of the final 60 min of the clamp was used as an estimate of insulin sensitivity^(25,31,32). Isotonic saline with K was continuously infused to avoid hypokalaemia. Blood for the analysis of insulin (every 30 min) was drawn into EDTA-containing tubes and centrifuged instantly. Plasma was stored at –80°C.

Lipopolysaccharide injection

Subjects received an intravenous bolus injection of *E. coli* LPS (0.3 ng/kg)^(33,34) on two experimental days, separated by the 4-week intervention period. On both days, subjects reported to the laboratory at 07.00 hours after an overnight fast and were immediately placed in bed. An intravenous catheter was placed in an antecubital vein for LPS injection and blood sampling. Non-invasive blood pressure, heart rate and tympanic temperature were monitored and recorded hourly throughout the trial. Blood samples were drawn at baseline and hourly until 8 h after LPS injection for subsequent plasma analyses. Plasma samples were stored at –80°C until further analyses.

Biochemical analyses

Blood samples for standard medical analyses obtained during the physical examination and plasma glucose during the OGTT were analysed at the Department of Clinical Biochemistry according to usual standards. Plasma glucose and K during the clamps were measured using an ABL555 (Radiometer, Copenhagen, Denmark). Plasma insulin was determined by ELISA (LOT 594148; DakoCytomation, Ely, Cambs, UK). C-reactive protein was measured with the Tina-quant C-reactive protein (latex) high sensitive assay (Roche Diagnostics Limited, Burgess Hill, West Sussex, UK). Plasma levels of TNF-α, IL-6 and IL-1 receptor antagonist (IL-1ra) were measured using ELISA (R&D Systems, Minneapolis, MN, USA). Measurements of plasma cytokines were done in duplicate and mean concentrations calculated. The ELISA intra-assay variation was evaluated by the CV of the duplicate measurements. Due to CV above 20%, 1% of the TNF measurements, 2.3% of the IL-6 and 1.2% of the IL-1ra measurements were discarded from further analyses. A CV of, respectively, 4.5, 5.4 and 4.0% was detected in the remaining TNF, IL-6 and IL-1ra measurements.

Statistical analyses

Data are presented as mean values and 95% CI with the exception of TNF, IL-6 and IL-1ra concentrations, which were log-transformed before analysis due to non-normal distribution, in order to obtain a better goodness of fit of the model. The estimated mean values and 95% CI of the cytokines were transformed back to the original scale, yielding geometric mean and 95% CI for the presentation of results.

Between-group variation in baseline characteristics was evaluated by a one-way ANOVA (SAS 9.1 Proc GLM; SAS Institute, Inc., Cary, NC, USA). Detection of *L. acidophilus* in faecal samples was evaluated by exact logistic regression. Changes in *M/I* and baseline inflammatory markers due to probiotic treatment were evaluated by applying a one-way ANOVA on the difference of these variables between the two study days, using treatment as the explanatory variable in the model (SAS 9.1 Proc GLM; model outcome (day 1 3– day 28) = treatment). If a significant effect of treatment was found, *post hoc t* tests were performed for subgroup analyses. The LPS-induced inflammatory responses before and after treatment were evaluated by applying an ANOVA for longitudinal measures (SAS 9.1, Proc Mixed) with time

(amount of hours post-LPS), study day (before or after treatment) and treatment (*L. acidophilus* NCFM or placebo) as explanatory variables, subject as a random factor and an autoregressive correlation matrix. The model was fitted on each investigated variable by backward regression. Goodness of fit of the models was assessed by evaluating the distribution of the residuals. If the ANOVA indicated a significant effect of time, Dunnett-adjusted *post hoc t* tests were subsequently performed to identify significant differences from baseline. Statistical software SAS 9.1 (SAS Institute, Inc.) was used throughout and $P < 0.05$ was considered to indicate statistical significance.

Results

Subjects

Of the twenty-four volunteers that were allocated to treatment with *L. acidophilus* NCFM, three subjects dropped out during the trial and data from these are consequently omitted from further analyses. Thus, ten subjects with NGT, three with IGT and eight with type 2 diabetes were treated with *L. acidophilus* NCFM ($n = 21$), while the placebo group included twelve volunteers with NGT, two with IGT and ten patients with type 2 diabetes ($n = 24$). The two treatment groups were comparable with regard to baseline characteristics as summarised in Table 1.

Detection of *Lactobacillus acidophilus* NCFM

Faecal samples from one subject in the *L. acidophilus* NCFM group were missing. Analyses were thus performed on twenty samples from participants treated with *L. acidophilus* NCFM and on twenty-four samples from the placebo group. In the group treated with *L. acidophilus*, it was detected in two out of twenty samples before and in fifteen out of twenty samples after the intervention ($P < 0.01$) (Table 2). Concomitantly, the concentration of *L. acidophilus* increased from undetectable levels in the samples collected before intervention to 6.4 (SD 0.7) colony-forming units per g stool after probiotic consumption. In the group treated with placebo ($n = 24$), *L. acidophilus* was detected in six samples before and three

samples after intervention (Table 2); this decrease was not statistically significant. No differences in *L. acidophilus* content were established between diabetic patients and individuals with NGT.

Insulin sensitivity

As evaluated by the one-way ANOVA, treatment allocation significantly affected the change in insulin sensitivity during the intervention period ($P < 0.01$) (Fig. 1). *Post hoc t* tests revealed that *MII* decreased in the placebo group ($P = 0.03$), indicating decreased insulin sensitivity, while there was a non-significant increase in *MII* in the *L. acidophilus* NCFM group ($P = 0.09$). When patients with or without type 2 diabetes were evaluated separately, we found the same trend in each of these groups as for the whole group. The variation in *MII* of each subject is depicted in Fig. 2. Of the subjects, one diabetic subject treated with *L. acidophilus* NCFM did not have a dual X-ray absorptiometry scan performed and fat-free body mass was therefore not determined on this subject. Due to the lack of information on fat-free body mass, an *MII* value could not be calculated, and clamp data on this subject was therefore not included in the statistical analysis.

Cytokines

The baseline characteristics and plasma cytokine concentrations measured during the injection of LPS before the intervention are reported as part of a separate paper, evaluating the differences in the inflammatory response between diabetic and non-diabetic volunteers⁽³⁵⁾.

In the present study, baseline plasma concentrations of TNF, IL-6, IL-1ra and C-reactive protein were comparable between the two treatment groups before the intervention and were unaffected by treatment with either *L. acidophilus* NCFM or placebo (Table 3).

The administration of LPS triggered a systemic inflammatory response with reversible increases in temperature, heart rate and leucocyte counts (data not shown), as well as in TNF, IL-6 and IL-1ra on both study days (Fig. 3(a–f)). LPS induced significant time-dependent changes in cytokines,

Table 1. Baseline characteristics

(Mean values and 95% confidence intervals)

	NGT ($n = 22$)		IGT ($n = 5$)		Diabetes ($n = 18$)		<i>Lactobacillus acidophilus</i> NCFM treatment ($n = 21$)‡		Placebo treatment ($n = 24$)‡	
	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
Age (years)	59	53, 64	62	60, 65	57	50, 63	55	48, 61	60	55, 66
BMI (kg/m^2)	27.6	25.5, 29.7	27.2	26.4, 28.0	29.4	26.0, 32.8	28.1	25.1, 31.1	28.7	26.1, 31.3
Fasting plasma glucose (mmol/l)	5.4	5.2, 5.7	5.9	5.7, 6.2	10.5*†	8.5, 12.6	8.0	5.8, 10.3	7.6	6.2, 9.0
HbA1c (%)	5.4	5.3, 5.6	5.9*	5.7, 6.1	7.8*†	6.7, 8.8	6.5	5.5, 7.5	6.5	5.8, 7.5
2h OGTT glucose (mmol/l)	5.1	4.5, 5.6	8.1*	7.8, 8.4	18.0*†	15.2, 20.8	11.9	7.6, 16.1	10.4	7.4, 13.3
HOMA2-IR	0.9	0.8, 1.1	1.4*	1.1, 1.7	2.0*†	1.5, 2.4	1.4	0.7, 2.2	1.5	1.1, 1.8

NGT, normal glucose tolerance; IGT, impaired glucose tolerance; OGTT, oral glucose tolerance test; HOMA2-IR, homeostatic model assessment of insulin resistance.

* Mean value was significantly different from that for the NGT group ($P < 0.05$).

† Mean value was significantly different from that for the IGT group ($P < 0.05$).

‡ The *L. acidophilus* NCFM group consisted of ten subjects with NGT, three with IGT and eight patients with type 2 diabetes, while twelve subjects with NGT, two with IGT and ten with type 2 diabetes were included in the placebo group. The two treatment groups were comparable.

Table 2. Number of faecal samples positive for *Lactobacillus acidophilus* detected by denaturing gradient gel electrophoresis *v.* total number of samples

	<i>L. acidophilus</i> NCFM treatment		Placebo treatment	
	Before	After	Before	After
Subjects with type 2 diabetes	2/20	15/20*	6/24	3/24

* Fractions before and after treatment differ significantly ($P < 0.01$).

which were comparable before and after the treatment course and independent of treatment allocation. Subgroup analyses confirmed these results both in diabetic and non-diabetic individuals.

Discussion

By use of a double-blinded, randomised setup, the present study demonstrates, compared with placebo, that 4 weeks treatment with the probiotic strain *L. acidophilus* NCFM appears to improve insulin sensitivity in a group consisting of subjects with NGT, IGT or type 2 diabetes. Since *L. acidophilus* NCFM treatment affected neither baseline inflammatory markers nor the induced inflammatory response, this effect seems not to be linked to the immune function.

In most of the faecal samples collected before the intervention, the concentration of *L. acidophilus* was below the detection limit of denaturing gradient gel electrophoresis PCR and quantitative PCR used in the present study. Thus, the increase both in the number of subjects positive for *L. acidophilus* and in the concentration of *L. acidophilus* after the intervention provided evidence of the presence of the

strain in significant amounts in the gastrointestinal tract. These results indicate a satisfactory compliance of participants, as well as an ability of the bacterium to survive gastrointestinal passage.

Subgroup analyses revealed that a consistent decrease in insulin sensitivity, which was absent in the *L. acidophilus* NCFM group, was evident among placebo-treated subjects. These results indicate an optimising effect of probiotic supplementation on insulin sensitivity, but the underlying mechanisms conveying this phenomenon remain unclear. A beneficial effect on glucose homeostasis is supported by a number of animal studies, in which specific probiotic strains have demonstrated anti-diabetic properties. Thus, antibiotic treatment may protect against diabetogenic changes in rodents, possibly by altering the composition of the gut microbiota^(36,37). Furthermore, a probiotic mixture of *L. acidophilus* and *L. casei* has been found to delay the onset of glucose intolerance, hyperglycaemia, dyslipidaemia and oxidative stress in rats with diet-induced diabetes^(2,3). Finally, probiotic supplementation improved high-fat diet-induced insulin resistance and hepatic steatosis⁽³⁸⁾. We cannot entirely exclude the possibility that the differences in insulin sensitivity between the two treatment groups in the present study are in fact caused by an unknown bias, hereby leading to a falsely positive result. The strict double-blinded, randomised design of the study as well as the absence of any obvious confounding factors do, however, make this unlikely. All participants were clamped using identical equipment and procedures

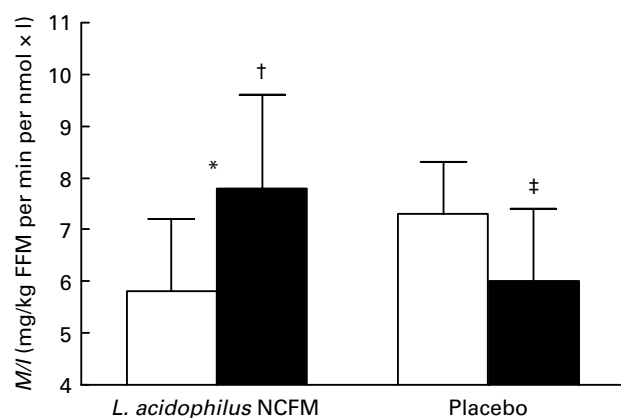


Fig. 1. Hyperinsulinaemic–euglycaemic clamps were performed in order to estimate insulin sensitivity, expressed as the mean ratio of the *M* value to plasma insulin concentrations (*M/I*) of the last 60 min of the clamp. *M/I* was calculated in forty-four males, randomised to intervention with either *Lactobacillus acidophilus* NCFM ($n = 20$, including ten subjects with NGT, three with IGT and seven with type 2 diabetes) or placebo ($n = 24$, including twelve subjects with NGT, two with IGT and ten with type 2 diabetes). The first clamp was performed at baseline (□) and the second clamp was performed after 4 weeks (28 d; ■) of intervention. Values are means, with 95% CI represented by vertical bars. * Change in *M/I* was significantly different from that for the placebo group ($P < 0.01$). † Mean value was marginally significantly different from that at day 1 ($P = 0.09$). ‡ Mean value was significantly different from that at day 1 ($P = 0.03$). FFM, fat-free body mass.

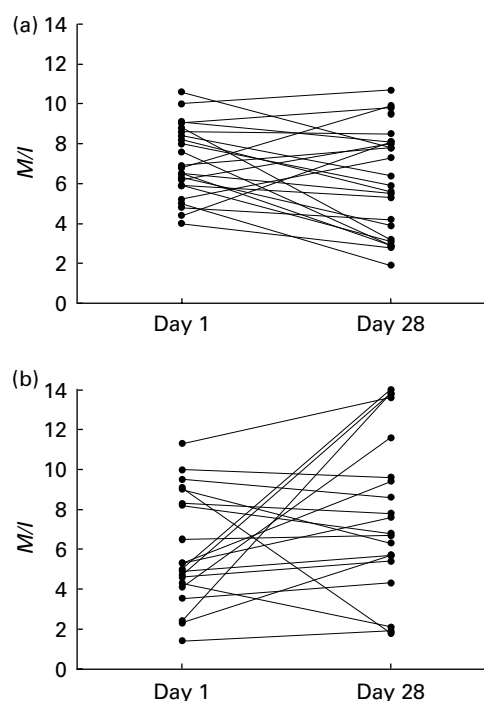


Fig. 2. Individual measures of insulin sensitivity, expressed as the mean ratio of the *M* value to plasma insulin concentration (*M/I*) values of the last 60 min of hyperinsulinaemic–euglycaemic clamps. *M/I* was calculated in forty-four males, randomised to intervention with either placebo (a) ($n = 24$; including twelve subjects with normal glucose tolerance (NGT), two with impaired glucose tolerance (IGT) and ten with type 2 diabetes) or *Lactobacillus acidophilus* NCFM (b) ($n = 20$; including ten subjects with NGT, three with IGT and seven with type 2 diabetes). The first clamp was performed at baseline and the second clamp was performed after 4 weeks (28 d) of intervention.

Table 3. Baseline plasma concentrations of inflammatory markers before and after 4 weeks' consumption of *Lactobacillus acidophilus* NCFM or placebo*

(Geometric mean values and 95 % confidence intervals)

Inflammatory markers	<i>L. acidophilus</i> NCFM treatment (n 21)				Placebo treatment (n 24)			
	Before		After		Before		After	
	Geometric mean	95 % CI	Geometric mean	95 % CI	Geometric mean	95 % CI	Geometric mean	95 % CI
TNF (pg/ml)	1.1	0.9, 1.3	1.1	0.9, 1.3	1.1	0.9, 1.4	1.2	1.0, 1.4
IL-6 (pg/ml)	1.5	1.1, 2.1	1.6	1.1, 2.3	1.7	1.4, 2.2	1.9	1.4, 2.5
IL-1ra (pg/ml)	246	177, 341	248	178, 345	244	200, 313	275	231, 330
CRP (mg/l)	1.3	0.7, 2.2	1.1	0.6, 1.9	1.5	0.9, 2.5	1.9	1.1, 3.3

IL-1ra, IL-1 receptor antagonist; CRP, C-reactive protein.

* Baseline concentrations were similar before and after the intervention, independent of treatment allocation.

before and after the intervention; participants were randomly allocated to either of the two treatment groups throughout the enrolment period; the allocation was double-blinded; the two treatment groups were comparable with regard to baseline data; anti-diabetic treatment of the diabetic patients was equally frequent in the two groups; and participants were

instructed to maintain their usual lifestyle throughout the study period. The most likely explanation of absence of decreased insulin sensitivity in the *L. acidophilus* NCFM group is therefore that a systematic decrease in insulin sensitivity was reversed by the supplementation of *L. acidophilus* NCFM.

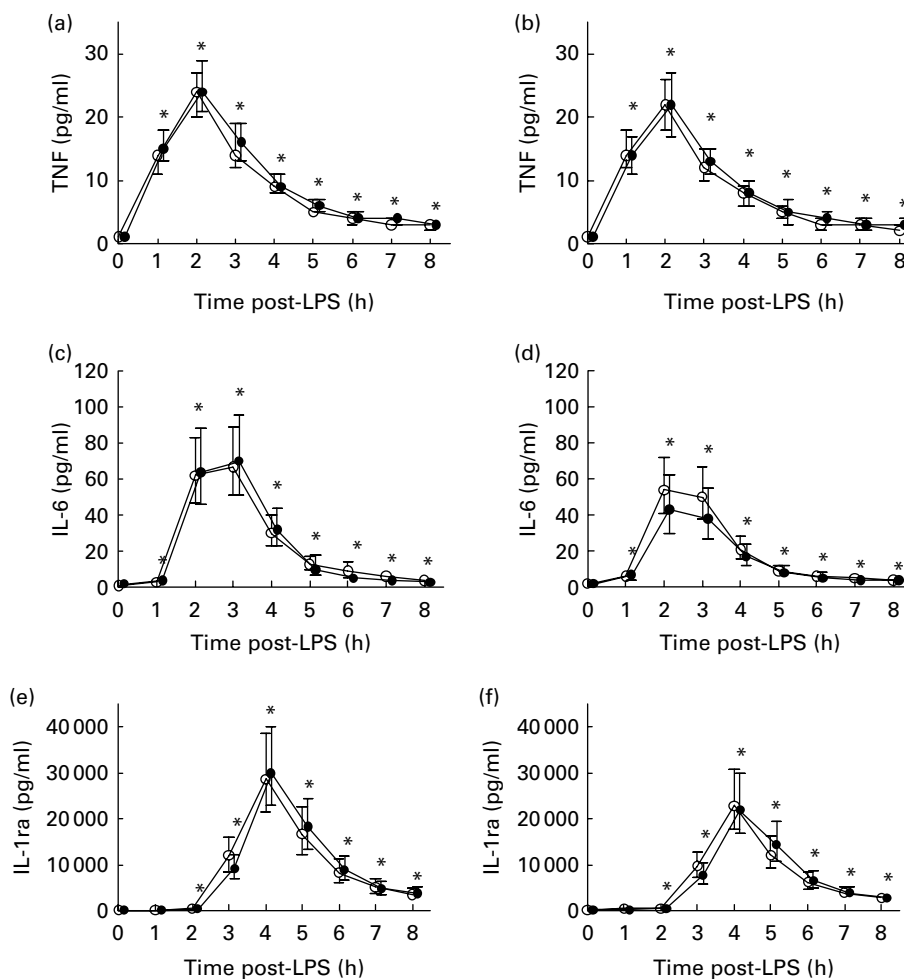


Fig. 3. An intravenous bolus injection of lipopolysaccharide (LPS) at a dose of 0.3 ng/kg was given to forty-five males (type 2 diabetes, impaired glucose tolerance, or normal glucose tolerance) before (○) and after (●) treatment with either *Lactobacillus acidophilus* NCFM (n 21; a, c, e) or placebo (n 24; b, d, f). Data are geometric means, with 95 % CI represented by vertical bars. According to the ANOVA, LPS induced significant changes in the plasma levels of TNF (a, b), IL-6 (c, d) and IL-1 receptor antagonist (IL-1ra) (e, f) in both treatment groups as well as before and after the intervention. However, the responses before and after treatment were similar, independent of treatment allocation. * Mean values were significantly different from those at baseline both before and after the intervention ($P < 0.05$).

We hypothesised that optimisation of insulin sensitivity due to probiotic treatment could be mediated, at least in part, through immune-modulatory effects. As mentioned above, previous studies have unveiled immune-modulatory and anti-inflammatory effects of *L. acidophilus* NCFM; *L. acidophilus* NCFM is capable of interacting with immune cells^(20,21) and improves intestinal integrity⁽²²⁾; consequently, *L. acidophilus* NCFM may diminish overflow of LPS from the gut to the systemic circulation and reduce the concomitant induction of Toll-like receptor (TLR)-4 signalling and pro-inflammatory cytokines. Furthermore, specific probiotic strains influence the *in vitro* secretion of cytokines from immune cells; hence, co-incubation of macrophage cultures with *Lactobacillus* species has been found to decrease the LPS-induced TNF release from macrophages⁽³⁹⁾. Since insulin resistance is notably associated with and induced by low-grade inflammation^(17–19,40), the beneficial effects of probiotic treatment on glucose homeostasis could potentially involve anti-inflammatory mechanisms. In the present study, TNF, IL-6, IL-1ra and C-reactive protein responses were unaffected by treatment allocation. The anti-inflammatory effects of probiotics are highly strain specific, possibly being related to surface antigens on the bacteria⁽⁴¹⁾, and the absent effect on inflammatory parameters in the present study could be due to the choice of bacterial strain. The observed improving effect on insulin sensitivity of *L. acidophilus* NCFM is thus evidently not related to its anti-inflammatory properties, at least not through any apparent effects on circulating cytokines, and our findings lend no support to the contention that insulin sensitivity is improved through probiotic-induced anti-inflammatory mechanisms. Alternatively, the variation in plasma cytokines due to the intervention may be so discreet that no significant changes during the treatment period were detected with the current number of volunteers included in the present study.

Altering the gut flora by means of probiotic treatment may convey beneficial effects upon insulin sensitivity through inflammation-dependent and -independent mechanisms⁽⁸⁾. Changes in circulating gut peptides and adipokines⁽⁸⁾, as well as adipose tissue growth⁽⁴²⁾ upon probiotic treatment, are likely to influence insulin sensitivity. Furthermore, gut microbiota influence the capacity of energy uptake from gut contents, and may thus have an impact on obesity^(9,43,44), which is a crucial risk factor for insulin resistance and type 2 diabetes^(19,45). However, even though long-term supplementation with certain probiotic strains potentially improves insulin sensitivity in type 2 diabetics through weight loss, the time scope for this explanation goes far beyond the settings of the present study. As of now, the mechanisms causing the beneficial effects of *L. acidophilus* NCFM on insulin sensitivity observed in the present study therefore remain unknown.

The present study is the first double-blinded, randomised, placebo-controlled trial investigating the effects of the widely applied and recommended probiotic strain *L. acidophilus* NCFM on insulin sensitivity in human subjects. Furthermore, it is the first of its kind to investigate the effect of a probiotic strain on the LPS-induced inflammatory response in a human *in vivo* model. This bacterium is generally believed to have beneficial effects on human health; in this context, the present results suggest that *L. acidophilus* NCFM may indeed 'confer health benefits on the host'.

In conclusion, compared with placebo, 4 weeks' intake of *L. acidophilus* NCFM preserves insulin sensitivity, but does not affect the systemic inflammatory response.

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A. S. A., T. P.-S. and R. B. carried out the study on the human volunteers. A. S. A. drafted the manuscript, the work involving the detection of *L. acidophilus* in the stool samples was performed by N. L. and K. D. S. provided statistical counselling. B. K. P., M. J. and K. M. conceived of the study and participated in its design and coordination and helped draft the manuscript. All authors read and approved the final manuscript.

There are no conflicts of interest.

References

1. Yadav H, Jain S & Sinha PR (2008) The effect of probiotic dahi containing *Lactobacillus acidophilus* and *Lactobacillus casei* on gastropathic consequences in diabetic rats. *J Med Food* **11**, 62–68.
2. Yadav H, Jain S & Sinha PR (2007) Antidiabetic effect of probiotic dahi containing *Lactobacillus acidophilus* and *Lactobacillus casei* in high fructose fed rats. *Nutrition* **23**, 62–68.
3. Yadav H, Jain S & Sinha PR (2008) Oral administration of dahi containing probiotic *Lactobacillus acidophilus* and *Lactobacillus casei* delayed the progression of streptozotocin-induced diabetes in rats. *J Dairy Res* **75**, 189–195.
4. Guarner F & Schaafsma GJ (1998) Probiotics. *Int J Food Microbiol* **39**, 237–238.
5. Food and Agriculture Organization & World Health Organization (2001) *Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria*. Geneva: WHO.
6. Marco ML, Pavan S & Kleerebezem M (2006) Towards understanding molecular modes of probiotic action. *Curr Opin Biotechnol* **17**, 204–210.
7. Jacobsen CN, Rosenfeldt Nielsen V, Hayford AE, *et al.* (1999) Screening of probiotic activities of forty-seven strains of *Lactobacillus spp.* by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. *Appl Environ Microbiol* **65**, 4949–4956.

8. Cani PD & Delzenne NM (2009) The role of the gut microbiota in energy metabolism and metabolic disease. *Curr Pharm Des* **15**, 1546–1558.
9. Turnbaugh PJ, Ley RE, Mahowald MA, *et al.* (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**, 1027–1031.
10. Jia W, Li H, Zhao L, *et al.* (2008) Gut microbiota: a potential new territory for drug targeting. *Nat Rev Drug Discov* **7**, 123–129.
11. Walker WA (2008) Mechanisms of action of probiotics. *Clin Infect Dis* **46**, Suppl. 2, S87–S91.
12. Rescigno M, Urbano M, Valzasina B, *et al.* (2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol* **2**, 361–367.
13. Guarner F & Malagelada JR (2003) Gut flora in health and disease. *Lancet* **361**, 512–519.
14. Madsen K, Cornish A, Soper P, *et al.* (2001) Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterology* **121**, 580–591.
15. Cani PD, Neyrinck AM, Fava F, *et al.* (2007) Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. *Diabetologia* **50**, 2374–2383.
16. Guha M & Mackman N (2001) LPS induction of gene expression in human monocytes. *Cell Signal* **13**, 85–94.
17. Pickup JC, Mattock MB, Chusney GD, *et al.* (1997) NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. *Diabetologia* **40**, 1286–1292.
18. Pickup JC (2004) Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. *Diabetes Care* **27**, 813–823.
19. Shoelson SE, Lee J & Goldfine AB (2006) Inflammation and insulin resistance. *J Clin Invest* **116**, 1793–1801.
20. Konstantinov SR, Smidt H, de Vos VM, *et al.* (2008) S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. *Proc Natl Acad Sci U S A* **105**, 19474–19479.
21. Chen CC, Chiu CH, Lin TY, *et al.* (2008) Effect of probiotics *L. acidophilus* on *C. rodentium* colitis: the role of dendritic cells. *Pediatr Res* (epublication ahead of print version 17 September 2008).
22. Ouwehand AC, Tiihonen K, Saarinen M, *et al.* (2009) Influence of a combination of *Lactobacillus acidophilus* NCFM and lactitol on healthy elderly: intestinal and immune parameters. *Br J Nutr* **101**, 367–375.
23. Sanders ME & Klaenhammer TR (2001) Invited review: the scientific basis of *Lactobacillus acidophilus* NCFM functionality as a probiotic. *J Dairy Sci* **84**, 319–331.
24. Altermann E, Russell WM, Azcarate-Peril MA, *et al.* (2005) Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. *Proc Natl Acad Sci U S A* **102**, 3906–3912.
25. DeFronzo RA, Tobin JD & Andres R (1979) Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol* **237**, E214–E223.
26. Andreasen AS, Krabbe KS, Krogh-Madsen R, *et al.* (2008) Human endotoxaemia as a model of systemic inflammation. *Curr Med Chem* **15**, 1697–1705.
27. World Health Organization (2006) *Definitions and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycemia. Report of a WHO/IIDF consultation*. Geneva: WHO.
28. Walter J, Hertel C, Tannock GW, *et al.* (2001) Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Appl Environ Microbiol* **67**, 2578–2585.
29. Muyzer G, de Waal EC & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**, 695–700.
30. ben Omar N & Ampe F (2000) Microbial community dynamics during production of the Mexican fermented maize dough pozol. *Appl Environ Microbiol* **66**, 3664–3673.
31. Ferrannini E & Mari A (1998) How to measure insulin sensitivity. *J Hypertens* **16**, 895–906.
32. Brehm A & Roden M (2009) Glucose clamp techniques. In *Clinical Diabetes Research, Methods and Techniques*, pp. 43–67 [M Roden, editor]. Chichester: John Wiley and Sons Ltd.
33. Taudorf S, Krabbe KS, Berg RM, *et al.* (2007) Human models of low-grade inflammation: bolus versus continuous infusion of endotoxin. *Clin Vaccine Immunol* **14**, 250–255.
34. Andreasen AS, Pedersen-Skovsgaard T, Mortensen OH, *et al.* (2009) The effect of glutamine infusion on the inflammatory response and HSP70 during human experimental endotoxaemia. *Crit Care* **13**, R7.
35. Andreasen AS, Pedersen-Skovsgaard T, Berg RMG, *et al.* (2009) Type 2 diabetes is associated with impaired cytokine response and adhesion molecule expression in human endotoxaemia. *Intensive Care Med* (epublication ahead of print version 13 March 2010).
36. Membrez M, Blancher F, Jaquet M, *et al.* (2008) Gut microbiota modulation with norfloxacin and ampicillin enhances glucose tolerance in mice. *FASEB J* **22**, 2416–2426.
37. Brugman S, Klatter FA, Visser JT, *et al.* (2006) Antibiotic treatment partially protects against type 1 diabetes in the Bio-Breeding diabetes-prone rat. Is the gut flora involved in the development of type 1 diabetes? *Diabetologia* **49**, 2105–2108.
38. Ma X, Hua J & Li Z (2008) Probiotics improve high-fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells. *J Hepatol* **49**, 821–830.
39. Diaz-Ropero MP, Martin R, Sierra S, *et al.* (2007) Two *Lactobacillus* strains, isolated from breast milk, differently modulate the immune response. *J Appl Microbiol* **102**, 337–343.
40. Hotamisligil GS, Arner P, Caro JF, *et al.* (1995) Increased adipose tissue expression of tumor necrosis factor- α in human obesity and insulin resistance. *J Clin Invest* **95**, 2409–2415.
41. Fink LN, Zeuthen LH, Ferlazzo G, *et al.* (2007) Human antigen-presenting cells respond differently to gut-derived probiotic bacteria but mediate similar strain-dependent NK and T cell activation. *FEMS Immunol Med Microbiol* **51**, 535–546.
42. Sato M, Uzu K, Yoshida T, *et al.* (2008) Effects of milk fermented by *Lactobacillus gasseri* SBT2055 on adipocyte size in rats. *Br J Nutr* **99**, 1013–1017.
43. Backhed F, Manchester JK, Semenkovich CF, *et al.* (2007) Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. *Proc Natl Acad Sci U S A* **104**, 979–984.
44. Backhed F, Ding H, Wang T, *et al.* (2004) The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci U S A* **101**, 15718–15723.
45. Hotamisligil GS (2006) Inflammation and metabolic disorders. *Nature* **444**, 860–867.