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Tolerance and safety of the potentially probiotic strain *Lactobacillus rhamnosus* PRSF-L477: a randomised, double-blind placebo-controlled trial in healthy volunteers

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In Europe, the species *Lactobacillus rhamnosus* is currently on the Qualified Presumption of Safety list used by the European Food Safety Authority (EFSA) for internal safety assessment, but according to the EFSA the species should remain a topic of surveillance. In the present study, the safety and tolerance of the potentially probiotic strain *L. rhamnosus* PRSF-L477 was investigated in a placebo-controlled double-blind volunteer trial following FAO/WHO guidelines. A total of thirty-four subjects received daily doses of 1×10^{11} colony-forming units (cfu) of *L. rhamnosus* PRSF-L477 (*n* 17) or placebo (*n* 17) for a period of 3 weeks, followed by a wash-out period of another 3 weeks. A questionnaire on gastrointestinal tolerance and a diary was kept daily to record compliance throughout these 6 weeks. Faecal and blood samples were collected for microbiological and haematological analysis. The recorded gastrointestinal symptoms, defecation frequency and stool consistency were not influenced indicating that *L. rhamnosus* PRSF-L477 was well tolerated. The species *L. rhamnosus* was detected in the faeces of sixteen out of seventeen subjects of the probiotic group during the intervention period. Using pulsed-field gel electrophoresis, re-isolates of *L. rhamnosus* PRSF-L477 were confirmed in nine of these subjects. Antibiotic susceptibility profiles of these re-isolates were unchanged compared with PRSF-L477. No clinically relevant changes in blood parameters such as liver and kidney function and no serious adverse events appeared during and after administration. Therefore, we conclude that *L. rhamnosus* PRSF-L477 can safely be administrated to healthy subjects at a daily dose of 1×10^{11} cfu.

***Lactobacillus rhamnosus*: Safety: Probiotics: Colonisation: Re-isolation: Antibiotic susceptibility**

Lactobacilli are commensal lactic acid bacteria of the human and animal gastrointestinal tract, but are also used worldwide as starter cultures in the production of dairy foods and as probiotics. Due to their long history of safe use in food applications, their very low pathogenicity and the low frequency of isolation from blood cultures, lactobacilli are generally regarded as safe in healthy subjects[1,2]. However, in a number of rare cases in immunocompromised patients or subjects with underlying diseases, *Lactobacillus* strains have been associated with cases of clinical infections such as bacteraemia and endocarditis. *Lactobacillus rhamnosus* and *L. paracasei* were most commonly isolated from such infections[3–5]. So far, a possible epidemiological link between probiotic consumption and rise in clinical isolates of lactobacilli has not been clearly established[6]. Nonetheless, several reports have linked infections directly to the consumption of probiotic products[7–10], but clinical trials and intervention studies have so far not indicated any safety problems with *L. rhamnosus* GG, LC705 or HN001[11–14]. However, conflicting results have been reported on the potential role of commensal lactic acid bacteria and bifidobacteria as vectors of antibiotic resistance elements in food and intestinal environments, which has triggered an ongoing debate[15–18]. As such, the fate of probiotic strains during gastrointestinal transit in an antibiotic-containing environment has previously been studied[19]. In the latter study a higher proportion of tetracycline-resistant anaerobically growing bacteria and bifidobacteria was detected in the antibiotic group compared with the control group. Several antibiotic-challenged subjects had faecal *Bifidobacterium animalis* subsp. *lactis* Bb-12-like isolates with reduced tetracycline resistance, which was, however, unlikely due to the acquisition of novel tetracycline resistance determinants. Recently, it has also been shown that a probiotic *Lactobacillus* strain can acquire vancomycin resistance during a digestive transit in mice[20].

It has been recommended by the European Food Safety Authority that *L. rhamnosus* remains in the Qualified Presumption of Safety list, but that this species should be

Abbreviations: BRCA, blood reinforced clostridial agar; cfu, colony-forming units; GSRS, Gastrointestinal Symptom Rating Scale; LAMVAB, *Lactobacillus* anaerobic MRS agar with vancomycin and bromocresol green; PFGE, pulsed-field gel electrophoresis; qPCR, quantitative real-time PCR.

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considered a topic of regular surveillance[21]. In their recommendations on biosafety assessment of probiotics used for human consumption based on the results of the European Union PROSAFE (Biosafety Evaluation of Lactic Acid Bacteria used for human consumption) project, participants from academics and industry recognised the relevance of human colonisation studies in a randomised placebo-controlled double-blind design, but did not specify which parameters should be determined in such studies[22]. For instance, limited attention has been given to the stability of a probiotic strain after passage through the gastrointestinal tract. Colonisation, but not stability, of *L. rhamnosus* strains has been studied through re-isolation from faecal samples[23–26].

In the present study, a placebo-controlled double-blind human volunteer trial was organised to investigate the tolerance and safety of a potentially probiotic *L. rhamnosus* strain, PRSF-L477, following WHO/FAO guidelines[27]. The study was performed as a part of the European Union PROSAFE project on the biosafety evaluation of lactic acid bacteria for human consumption. PRSF-L477 has been proposed to modulate dendritic cell function to induce a novel form of T cell responsiveness. An anti-inflammatory mechanism of PRSF-L477 has been proposed and therefore it may be of interest for the treatment of a variety of inflammatory disorders[28]. PRSF-L477 may also be of benefit in the preservation of gut barrier integrity after injury or stress, as has been shown previously in a rat model for haemorrhagic shock[29]. From the host's perspective, evaluation of tolerance and safety in the present study was based on questionnaires, blood safety parameters and the number and type of adverse events. Also, colonisation and wash-out kinetics of *L. rhamnosus* PRSF-L477 were determined, and the phenotypic and genotypic stability of faecal re-isolates of this strain from healthy subjects was assessed.

Experimental methods

Study population

A total of thirty-six healthy volunteers were enrolled in the study using the following exclusion criteria: pregnancy and breast-feeding; blood parameters outside the normal range and considered clinically significant; history of metabolic or gastrointestinal disease; food allergies; recent use of antibiotics or laxative drugs; diarrhoea; constipation; diabetes mellitus; blood pressure $>$ 150/90 mmHg. The inclusion criteria were: healthy male or female; age 18–60 years; normal defecation pattern; blood parameters within the normal range or not considered clinically significant if outside the normal range; BMI 18·0–29·9 kg/m^2; written informed consent.

Trial design

The study was a randomised, placebo-controlled, double-blind, parallel-group study. The study was conducted according to guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the ethics review committee of the Institutional Review Board Nijmegen (The Netherlands). After having signed informed consent, subjects were screened for eligibility into the study. Subjects taking probiotic preparations were asked to enter a 3-week wash-out period before screening (Fig. 1). Subjects eligible for participation (*n* 36) were randomly allocated to receive either the probiotic or the placebo product after stratification for age ($<$40 years and $>$ 40 years). Subjects were instructed to take the product for a period of 3 weeks, followed by a wash-out period of another 3 weeks. Faecal samples were collected weekly throughout these 6 weeks, whereas a blood sample was taken at screening and after 3 weeks. Questionnaires were filled out after 3 and 6 weeks. A diary was kept daily from screening onwards until the end of the study. A double data entry procedure was used. The study was conducted between September 2005 and December 2005 by the former Menox B.V., currently known as Ampha B.V. (Nijmegen, The Netherlands). Regular quality checks were performed by the study monitor.

Study parameters

The primary objective of the study was to assess the stability of *L. rhamnosus* PRSF-L477 in the gut of healthy subjects. The secondary objective was to assess the gastrointestinal tolerance and safety of *L. rhamnosus* PRSF-L477. Sample size was calculated based on the primary study parameter.

Product description

The probiotic study product contained lyophilised *L. rhamnosus* PRSF-L477 in a total daily dose of 1×10^{11} colony-forming

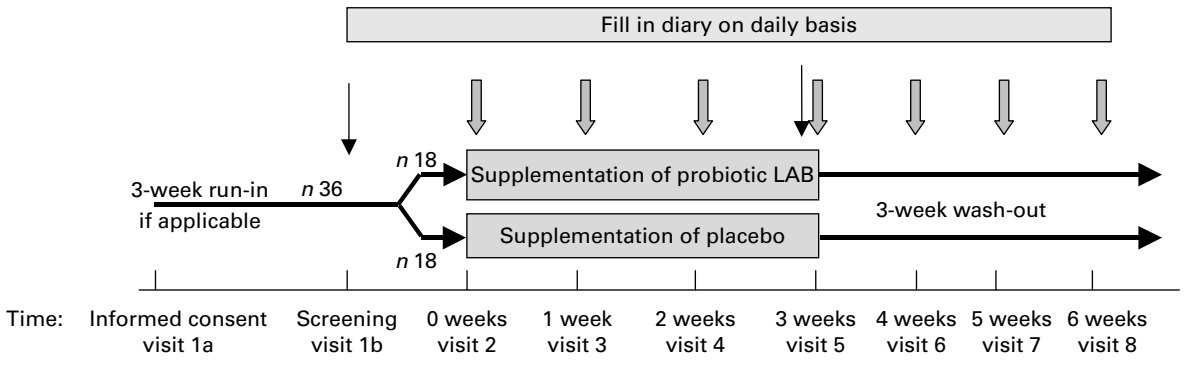

Fig. 1. Study design. LAB, lactic acid bacteria; ⇩, stool sample; ↓, blood sample.

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units (cfu) (Numico Research Culture Collection, Wageningen, The Netherlands). The probiotic product was provided in sachets by Milupa GmbH (Fulda, Germany). Subjects were instructed to take two sachets per d. Each sachet contained 0·5 g lyophilised PRSF-L477 at 1×10^{11} cfu/g and was filled up with maltodextrin to 7·5 g in total. The placebo product contained 7·5 g maltodextrin and was designed to have a similar appearance and taste as the probiotic product.

Microbiological determination

Faecal samples were analysed by plating appropriate dilutions on blood reinforced clostridial agar (BRCA) for the total number of anaerobic bacteria and *Lactobacillus* anaerobic de Man–Rogosa–Sharpe (MRS) agar with vancomycin and bromocresol green (LAMVAB) agar for the number of lactobacilli[30,31]. BRCA contains reinforced clostridial medium (38 g/l) (Oxoid, Basingstoke, Hants, UK), agar bacteriological (18 g/l) (Oxoid), vitamin K_1 (three drops/l) (0·25 mg/g; Pharmacy Churchill, Wageningen, The Netherlands), defibrinated sheep blood (50 ml/l) (bioTrading, Mijdrecht, The Netherlands) and salt solution (40 ml/l) containing $MgSO_4$ (0·2 g/l), $CaCl_2$ (0·2 g/l), K_2HPO_4 (1·0 g/l), KH_2PO_4 (1·0 g/l), $NaHCO_3$ (10 g/l) and NaCl (2·0 g/l). LAMVAB contains MRS (52 g/l) (Oxoid), L-cysteine-HCl (0·25 g/l) (Sigma-Aldrich, St Louis, MO, USA), broomcresol green (0·025 g/l) (Merck, Darmstadt, Germany), agar bacteriological (20 g/l) (Oxoid) and vancomycin-HCl (20 mg/l) (Pharmacy Churchill, Wageningen, The Netherlands). The number of *L. rhamnosus* was determined on modified LAMVAB, in which glucose was substituted by rhamnose and the antibiotics kanamycin and colistin sulfate (both 62 mg/l) were added. Only the species *L. rhamnosus* and a few other *Lactobacillus* species that are less common or even not present in the human gastrointestinal tract are able to grow on this medium[32]. The total number of bacteria was determined with fluorescent *in situ* hybridisation as previously described[33]. The pH of the faecal samples was measured to determine the influence of PRSF-L477 supplementation on faecal pH values.

Re-isolation procedure

Faecal samples were plated on modified LAMVAB agar plates for re-isolation of *L. rhamnosus* PRSF-L477. Five to ten colonies grown on the modified LAMVAB medium from plated faecal samples, preferably as late as possible in the study, were picked and their identity was determined with quantitative real-time PCR (qPCR) specific for *L. rhamnosus* [34] and a qPCR specific for *L. rhamnosus* cluster VII (PRSF-L477 and closely related *L. rhamnosus* [35]). The MGB-Taqman probe used for *L. rhamnosus* cluster VII was prepared by Biolegio (Malden, The Netherlands) (CCT GGA CAC ACG AAA); primers used were the same as described earlier by Haarman & Knol for *L. rhamnosus* [34]. The species identity of the re-isolates obtained from the probiotic group and positive for the cluster VII qPCR was confirmed with 16S rDNA sequencing (Baseclear, Leiden, The Netherlands) and strain identity by PFGE.

Characterisation of PRSF-L477 re-isolates

The original strain PRSF-L477 and a selection of re-isolates were subjected to pulsed-field gel electrophoresis (PFGE) using *Not*I as a restriction enzyme according to the protocol of Vancanneyt *et al.*[35]. All isolates were analysed twice with PFGE analysis. Conversion, normalisation and analysis of the band patterns were performed using Bionumerics software version 4.01 (Applied Maths, Sint-Martens Latem, Belgium). Correlation coefficients and levels of similarity were calculated using the Dice coefficient and cluster analysis using the unweighted pair group method.

Antibiotic susceptibility

The susceptibilities of the original strain PRSF-L477 and a selection of re-isolates to nineteen antimicrobials were tested by determination of minimal inhibitory concentrations in broth microdilution using LSM broth, which is a mixture of IsoSensitest broth (90 %) and de Man–Rogosa–Sharpe broth (10 %) adjusted to pH 6·7 and supplemented with L-cysteine hydrochloride (0·3 g/l)[36].

Platelet aggregation

Aggregation was measured in an aggregometer (Payton Lumi-aggregometer module series 100B; Payton Scientific, Buffalo, NY, USA), by changes in the transmission of the platelet suspension. Standard buffy coat-derived platelet concentrates in plasma were prepared at Sanquin Research (Amsterdam, The Netherlands) from whole blood units collected by Sanquin Blood Bank and pooled from five different donors. Bacterial strains were used as liquid stationary phase cultures in PBS at pH 6·7. The platelet concentrates were diluted with plasma to a concentration of $2{\cdot}5 \times 10^8$ cells/ml. For the various tests, 10 % (v/v) of a bacterial suspension at a concentration of $2{\cdot}5 \times 10^9$ cells/ml was added. The aggregation curve was followed for 25 min as previously described[37].

Gastrointestinal tolerance and safety parameters

Gastrointestinal tolerance was determined with the Gastrointestinal Symptom Rating Scale (GSRS)[38], the King's Stool Chart for stool consistency[39], the daily recorded gastrointestinal symptoms of nausea, vomiting, diarrhoea, burping, abdominal distension and flatulence[40] and the defecation frequency. The GSRS and stool consistency were measured by the investigator at baseline and after 3 and 6 weeks. Product compliance was recorded daily in a diary. Intolerance was defined as a symptom score of 2 or higher (moderate or severe) on the GSRS. Safety parameters were the number and type of adverse events recorded throughout the whole study and a change from baseline blood parameters determined at the end of the supplementation period. Measurement of blood parameters was performed by a clinical laboratory (Stichting Huisartsenlaboratorium Oost (SHO), Velp, The Netherlands) and included haematological parameters (erythrocyte sedimentation rate (mm/h), Hb (mmol/l), packed cell volume (l/l), cholesterol (mmol/l), HDL-cholesterol (mmol/l), glucose (mmol/l), mean corpuscular volume (fl) and leucocytes ($\times 10^9$/l)), and parameters for liver function (aspartate

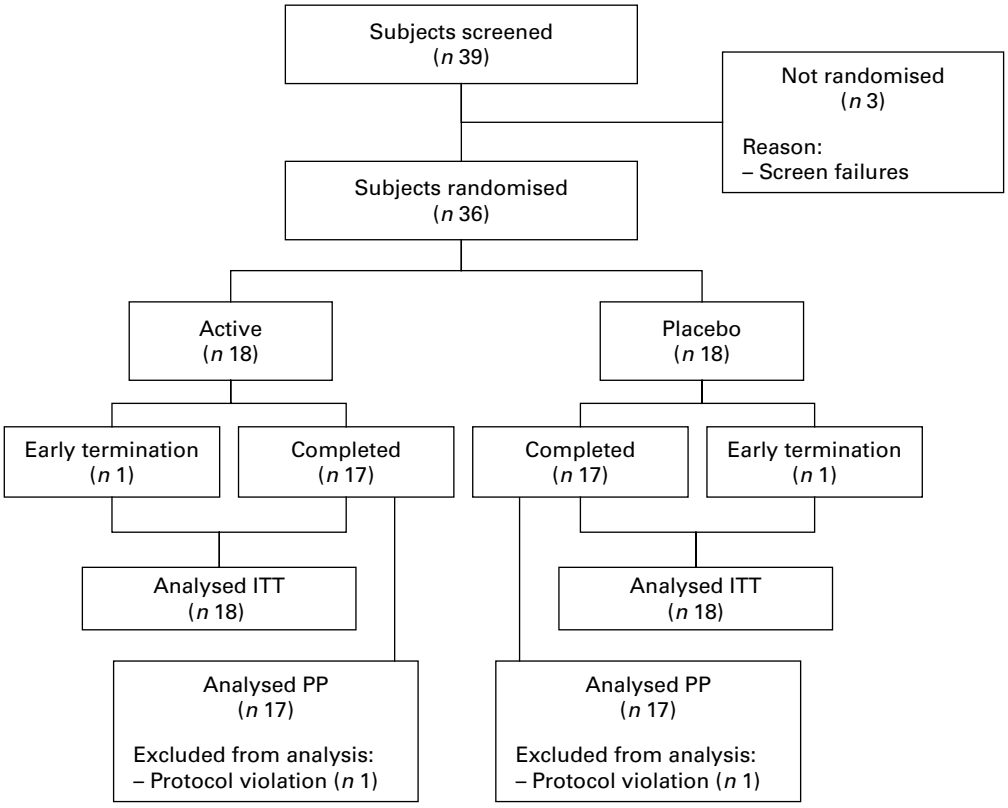

Fig. 2. Flow chart of the study subjects. ITT, intention to treat; PP, per protocol.

aminotransaminase (IU/l), alanine aminotransaminase (IU/l), γ-glutamyl transferase (U/l)) and kidney function (creatinine (μmol/l)).

Statistics

Statistical analyses of gastrointestinal symptom scores were performed using the Mann–Whitney *U* test for equivalence. Other parameters were tested for normal distribution with homogeneous variance. Normally distributed parameters were tested for statistically significant differences using the *t* test or paired *t* test, while the Mann–Whitney *U* test or paired Wilcoxon test was used for not normally distributed parameters.

Results

Subjects

A total of thirty-four subjects aged 18–60 years completed the study, whereas two female subjects left the study group prematurely because of antibiotic use (Fig. 2). Except for the fact that more women than men participated in the study (61 *v.* 39 %), subjects were well balanced over the two study groups with respect to baseline characteristics such as age, height, weight, BMI, heart rate and blood pressure (Table 1). The median age for female and male subjects was 51 and 43 years, respectively, whereas the median age for the whole study population was 47 years. Product compliance in the

Table 1. Baseline characteristics of the study groups (per-protocol population)
(Mean values and standard deviations)

	Total group (*n* 34)		Probiotic group (*n* 17)		Placebo group (*n* 17)	
	Mean	SD	Mean	SD	Mean	SD
Sex (*n*)						
Male	14		8		6	
Female	20		9		11	
Age (years)	42	16	40	18	44	14
Height (m)	1·73	0·12	1·74	0·12	1·72	0·12
Weight (kg)	70·6	13·4	71·2	10·9	70·0	15·8
BMI (kg/m^2)	23·5	3·1	23·6	3·0	23·4	3·3
Heart rate (beats/min)	63	11	61	12	66	8
Blood pressure (mmHg)						
Systolic	120	13	118	11	121	16
Diastolic	75	7	73	7	76	7

Table 2. Gastrointestinal symptom score according to the Gastrointestinal Symptom Rating Scale (GSRS) in the per-protocol population
(Median values and ranges)

	GSRS symptom score*											
	Probiotic group						Placebo group					
	Before supplementation		After supplementation		After follow-up		Before supplementation		After supplementation		After follow-up	
Symptom	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
Abdominal pains (q1)	0		0	0–1	0	0–1	0		0		0	
Heartburn (q2)	0	0–1	0	0–1	0	0–1	0	0–1	0	0–1	0	0–1
Acid regurgitation (q3)	0	0–1	0	0–1	0	0–1	0		0	0–1	0	0–1
Sucking sensations in the epigastrium (q4)	0		0	0–1	0		0		0	0–1	0	
Nausea and vomiting (q5)	0	0–1	0	0–1	0	0–1	0	0–1	0		0	
Borborygmus (q6)	0	0–1	0	0–1	0	0–1	0	0–1	0	0–2	0	
Abdominal distension (q7)	0	0–1	0	0–1	0	0–1	0	0–1	0	0–1	0	0–1
Eructation (q8)	0	0–1	0	0–1	0	0–1	0	0–1	0	0–1	0	0–1
Loose stools (q12)†	0	0–1	0	0–1	0	0–1	0	0–1	0	0–1	0	0–1
Hard stools (q13)†	0·5	0–1	0	0–1	0	0–2	0		0	0–1	0	0–1
Urgent need for defecation (q14)	0	0–1	0	0–1	0	0–1	0	0–2	0	0–2	0	0–1
Feeling of incomplete evacuation (q15)	0	0–1	0	0–1	0	0–1	0	0–1	0		0	0–1
Dyspeptic syndrome (q1–5)	0	0–0·40	0	0–0·80	0	0–0·80	0	0–0·20	0	0–0·40	0	0–0·40
Indigestion syndrome (q6–8)	0	0–0·67	0	0–1·00	0	0–0·67	0	0–1·00	0	0–0·67	0	0–0·33
Bowel dysfunction syndrome (q12–15)	0	0–1·00	0·17	0–0·83	0	0–0·83	0	0–0·83	0	0–0·83	0	0–0·58

*0 = absent; 1 = mild; 2 = moderate; 3 = severe.
† Either question (q) 12 or q 13 was answered.

group of subjects included in the per-protocol analysis was 95–100 % for all subjects, except for one individual in the placebo group who had a compliance of 90–95 %.

Gastrointestinal tolerance and safety parameters

Symptom scores as measured by the GSRS questionnaire were all less than 2 and there was no significant difference between the control group and supplemented group (Table 2). The median score of the daily recorded gastrointestinal symptoms of nausea, vomiting, diarrhoea, abdominal distension, burping and flatulence did not change during the probiotic supplementation period and the follow-up period. There were no median scores of 2 or higher. Also, the stool consistency and defecation frequency did not change during the supplementation period and the follow-up period in both the probiotic and placebo groups. No serious adverse events during the supplementation period in either the placebo or probiotic group were observed. In the probiotic group, sixteen adverse events were reported, of which six were judged as possibly related to the study product: increased flatulence (*n* 2), intermittent abdominal cramps (*n* 1), more loose stools (*n* 1), pain in the lower abdomen (*n* 1) and sensitive lower abdomen minor pain (*n* 1). In the placebo group, twenty-seven adverse events were reported, of which five were possibly related to the study product: looser stools (*n* 4) and stinging pain in the lower abdomen (*n* 1). In the GSRS questionnaire the difference between the probiotic and placebo groups was not significant on the symptoms of abdominal pains and loose stools (Table 2).

Blood parameters for testing the safety of *L. rhamnosus* PRSF-L477 administration were measured before and at the end of the supplementation period. No clinically relevant changes in blood safety parameters were found (Table 3). Creatinine tended to decrease in the probiotic group and to increase in the placebo group during the supplementation period. These changes in creatinine concentration were not considered to be clinically relevant by the medical study monitor, as reference values used for adult men and women were between 50 and 125 μmol/l. Moreover, all creatinine concentrations of individual subjects were within the reference values ranging from 62 to 111 μmol/l before and from 60 to 103 μmol/l after supplementation. There were no significant differences in the other blood parameters between the probiotic and placebo group before and at the end of the supplementation period.

Re-isolation of Lactobacillus rhamnosus *PRSF-L477*

L. rhamnosus PRSF-L477 was re-isolated from the collected faecal samples. One faecal re-isolate from each subject of the probiotic group (*n* 17) that was positive for *L. rhamnosus* and for *L. rhamnosus* cluster VII-specific qPCR was further identified with 16S rDNA sequencing. *L. rhamnosus* was detected in the faeces of sixteen out of seventeen subjects of the probiotic group during the supplementation and follow-up period. This single non-responder was shown to be low in lactobacilli and *L. rhamnosus* counts during the whole study period. From these sixteen positive subjects, identification as *L. rhamnosus* was confirmed by 16S rDNA sequencing for twelve faecal re-isolates from eleven different subjects. PFGE analysis indicated that nine out of these twelve re-isolates exhibited fingerprints that were indistinguishable from the profile of PRSF-L477 (Fig. 3). The remaining three

Table 3. Blood parameters in the per-protocol population
(Mean values with their standard errors)

	Concentration							
	Probiotic group				Placebo group			
	Before supplementation		After supplementation		Before supplementation		After supplementation	
Blood parameter	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Haematological								
ESR (mm/h)	4	1	4	1	6	1	7	2
Hb (mmol/l)	8·9	0·2	8·9	0·2	8·9	0·2	8·9	0·2
Packed cell volume (l/l)	0·42	0·01	0·42	0·01	0·43	0·01	0·42	0·01
Liver function								
ASAT (IU/l)	21	2	20	1	18	2	19	2
ALAT (IU/l)	24	4	24	4	24	3	26	4
γ-GT (U/l)	24	2	23	3	23	3	24	4
Kidney function								
Creatinine (μmol/l)	81	3	78*	3	80	3	82*	2
Other								
Cholesterol (mmol/l)	5·2	0·3	5·2	0·3	5·2	0·3	5·1	0·3
HDL-cholesterol (mmol/l)	1·27	0·07	1·27	0·08	1·31	0·06	1·30	0·07
Cholesterol:HDL-cholesterol ratio	4·2	0·3	4·3	0·3	4·1	0·3	4·0	0·3
Glucose (mmol/l)	5·2	0·1	5·2	0·1	5·4	0·1	5·3	0·1
MCV (fl)	91	1	91	1	92	1	92	1
Leucocytes ($\times 10^9$/litre)	5·4	0·3	5·4	0·2	5·8	0·4	5·8	0·4

ESR, erythrocyte sedimentation rate; ASAT, aspartate aminotransaminase; ALAT, alanine aminotransaminase, γ-GT, γ-glutamyl transferase; MCV, mean corpuscular volume.
* Statistical trend between probiotic and placebo group with regard to before supplementation (P=0·081; t test).

Fig. 3. Pulsed-field gel electrophoresis (*Not*I) profiles of *Lactobacillus rhamnosus* PRSF-L477 and its faecal re-isolates after gastrointestinal tract passage. The designation code of the re-isolates comprises information on subject number, sample number and colony number, respectively.

isolates, i.e. 101-4D, 209-5C and 216-4D, showed a completely different PFGE profile compared with the administered *L. rhamnosus* PRSF-L477, indicating that they were not true re-isolates of PRSF-L477. No positive re-isolates of *L. rhamnosus* PRSF-L477 were found in the faecal samples of the probiotic group collected during the wash-out period.

In the placebo group re-isolates of *L. rhamnosus* were detected by plating and qPCR in ten out of seventeen subjects; these were, however, not further identified by 16S rDNA sequencing or PFGE analysis.

Antibiotic susceptibility profiling

Antibiotic susceptibility profiling indicated that the three isolates that displayed a completely different PFGE profile compared with PRSF-L477 (i.e. 101-4D, 209-5C and 216-4D) also exhibited a different antibiotic susceptibility profile (Table 4). The other nine re-isolates showed a highly comparable if not identical antibiotic susceptibility profile to PRSF-L477. Both the supplemented PRSF-L477 and re-isolate 110-4E were negative for platelet aggregation within 25 min. Other re-isolates were not tested for platelet aggregation.

Colonisation and wash-out kinetics

The viable count of total anaerobic bacteria, lactobacilli and the species *L. rhamnosus* was determined by plating on respectively BRCA, LAMVAB and modified LAMVAB agar, before and during the supplementation period and

Table 4. Minimal inhibitory concentrations (MIC) to different antimicrobials for *Lactobacillus rhamnosus* PRSF-L477 and its faecal re-isolates

	MIC (mg/l) to the antimicrobials*														
Designation	PEN	AMP	ASU	GEN	STR	VAN	TPL	Q/D	ERY	CLI	OTE	CMP	FUS	TMP	SXT	LNZ
PRSF-L477 probiotic	0·5	1	1	⩽1	⩽2	>256	>256	0·25	0·063	0·125	0·25	2	64	16	128	1
PRSF-L477 101-4D	0·25	0·5	0·5	2	4	>256	>256	0·125	0·032	0·063	0·125	2	64	4	32	1
PRSF-L477 110-4E	0·25	0·5	0·5	⩽1	⩽2	>256	>256	0·25	0·063	0·125	0·25	2	64	16	128	1
PRSF-L477 113-2E	0·25	0·5	0·5	⩽1	4	>256	>256	0·25	0·032	0·063	0·25	2	64	16	128	1
PRSF-L477 201-4C	0·25	0·5	0·5	⩽1	⩽2	>256	>256	0·25	0·063	0·125	0·25	2	128	16	256	1
PRSF-L477 202-4A	0·5	1	1	⩽1	⩽2	>256	>256	0·25	0·063	0·125	0·25	2	128	64	256	1
PRSF-L477 208-4D	0·25	1	1	⩽1	⩽2	>256	>256	0·25	0·063	0·125	0·25	2	128	32	128	1
PRSF-L477 209-4B	0·5	1	1	⩽1	⩽2	>256	>256	0·25	0·063	0·125	0·25	2	128	16	128	1
PRSF-L477 209-5C	0·5	1	1	2	16	>256	>256	0·25	0·063	0·063	0·25	2	64	16	128	1
PRSF-L477 211-2A	0·5	1	1	⩽1	⩽2	>256	>256	0·25	0·063	0·063	0·25	2	64	16	128	1
PRSF-L477 216-4D	0·25	0·5	0·5	⩽1	4	>256	256	0·25	0·032	⩽0·032	0·25	2	64	⩽0·25	2	0·5
PRSF-L477 218-4E	0·25	0·5	0·5	⩽1	⩽2	>256	>256	0·25	0·032	0·125	0·25	2	128	16	128	1
PRSF-L477 221-4E	0·25	0·5	0·5	⩽1	⩽2	>256	>256	0·25	0·032	0·063	0·25	2	128	16	128	1

PEN, penicillin G; AMP, ampicillin; ASU, ampicillin/sulbactam; GEN, gentamicin; STR, streptomycin; VAN, vancomycin; TPL, teicoplanin; Q/D, quinupristin-dalfopristin; ERY, erythromycin; CLI, clindamycin; OTE, oxytetracycline; CMP, chloramphenicol; FUS, fusidic acid; TMP, trimethoprim; SXT, sulfamethoxazole/trimethoprim; LNZ, linezolid.

*Deviations of ± one $\log_2$ dilution step correspond to the standard deviation of this microbiological antibiotic susceptibility test (broth microdilution).

Table 5. Plate counts (log colony-forming units/g wet faeces) for the different bacterial groups from faecal samples collected at several time points of the study for the entire population (per-protocol population)

(Mean values and standard deviations)

	t = 0 weeks		t = 1 week		t = 3 weeks		Wash-out (4 weeks)	
Bacteria	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Total anaerobic count (BRCA)								
Probiotic group	8·7	0·7	8·7	0·4	8·5	0·5	8·5	0·6
Placebo group	8·4	0·5	8·4	0·7	8·5	0·6	8·6	0·6
Lactobacilli (LAMVAB)								
Probiotic group	3·6	1·3	4·5*	1·1	4·5*	1·1	3·7‡	1·7
Placebo group	4·2	1·0	3·8†	1·6	3·6§	1·2	3·7‖	1·1
Lactobacillus rhamnosus (modified LAMVAB)								
Probiotic group	3·0	1·4	4·0*	1·0	3·9*	1·4	3·1‡	1·6
Placebo group	3·5	1·4	3·1†	1·6	3·0†	1·4	3·1¶	1·2

BRCA, blood reinforced clostridial agar; LAMVAB, *Lactobacillus* anaerobic MRS agar with vancomycin and bromocresol green.

* Significant difference compared with t = 0 weeks ($P<0·05$; paired *t* test).

† Non-significant difference compared with t = 0 weeks ($P>0·05$; paired Wilcoxon test).

‡ Non-significant difference compared with t = 0 weeks ($P>0·05$), significant wash-out compared with t = 3 weeks ($P<0·05$) (paired Wilcoxon test).

§ Significant difference compared with t = 0 weeks ($P<0·05$; paired Wilcoxon test).

‖ Non-significant difference compared with t = 0 weeks ($P>0·05$; paired Wilcoxon test), non-significant difference compared with t = 3 weeks ($P>0·05$; paired *t* test).

¶ Non-significant difference compared with t = 0 and t = 3 weeks ($P>0·05$; paired Wilcoxon test).

1 week after the end of the supplementation period (Table 5). During the supplementation period, the number of lactobacilli and *L. rhamnosus* in the faeces showed a significant increase in the probiotic group ($P<0·05$; paired *t* test). In the placebo group both the number of lactobacilli and *L. rhamnosus* decreased during the supplementation period. This decrease was non-significant except for the number of lactobacilli in the faecal samples after 3 weeks compared with the start of the study (paired Wilcoxon test). There was already a significant difference in the number of *L. rhamnosus* between the probiotic and the placebo group after 1 week of supplementation ($P=0·012$; *t* test). During the follow-up period, the number of lactobacilli and *L. rhamnosus* decreased to initial levels in the probiotic group within 1 week after supplementation had stopped, while in the placebo group counts remained stable at the lower levels found in week 1. There were no significant differences in the total number of bacteria determined by fluorescent *in situ* hybridisation as median values ranged from 10·7 to 10·9 log cfu/g wet faeces with a standard deviation of 0·2–0·3 ($P>0·05$; *t* test). There were also no significant differences in the total number of anaerobic bacteria determined by plating between the probiotic and placebo groups at any time point during the study ($P>0·05$; Mann–Whitney *U* test). The pH of the faeces did not change during the supplementation period and the follow-up period in both the probiotic and placebo groups (data not shown).

Discussion

In the present study, the tolerance, safety, colonisation and stability of the potential probiotic strain *L. rhamnosus* PRSF-L477 were investigated in healthy volunteers. Administration of a daily dose of 1×10^{11} cfu *L. rhamnosus* PRSF-L477 was safe and well tolerated in the population tested. The recorded gastrointestinal symptoms (GSRS and daily recorded symptoms), defecation frequency and stool consistency were not influenced. No clinically relevant changes in blood parameters and no serious adverse events appeared during and after administration. In addition, it has been suggested that the safety of probiotics should be tested by the detection of undesirable changes in immune parameters[41] or unwanted changes in harmful faecal enzyme activities[42]. These tests were not included in the present study, indicating that various other parameters may need to be included in both short-term and long-term safety evaluations before applying new *L. rhamnosus* strains in more vulnerable target populations such as, for example, severely immune-compromised individuals.

The administered strain *L. rhamnosus* PRSF-L477 was re-isolated from nine subjects in the probiotic group during the supplementation period. The PFGE and the antibiotic susceptibility profiles of the faecal re-isolates remained virtually unchanged compared with the originally administered strain during passage through the gastrointestinal tract of healthy subjects. Solely based on antibiotic susceptibility profiling of nine faecal re-isolates, these data suggest that *L. rhamnosus* PRSF-L477 was phenotypically stable during the study period. In terms of risk assessment, more research including a higher number of re-isolates would be needed to investigate the possible transfer of resistance genes from the autochthonous gut microbiota to strain PRSF-L477 in healthy volunteers. In another study where tetracycline susceptibility of ingested *L. acidophilus* LaCH-5 and *B. animalis* subsp. *lactis* Bb-12 during antibiotic-probiotic intervention was investigated, oral tetracycline therapy resulted in increased tetracycline resistance among faecal anaerobic bacteria including the ingested Bb-12 strain[19]. When Bb-12-like isolates were subcultured again without antibiotic selection, tetracycline minimal inhibitory concentrations decreased but not

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to the original level. From the study it was, however, not possible to say whether this was due to changes that had occurred in the *tet*(W) gene or to its regulation in these isolates during antibiotic therapy.

In a previous study, a set of 118 *L. rhamnosus* strains intended for probiotic use and isolates of human origin were genotypically characterised using amplified fragment length polymorphism (AFLP) and PFGE[35]. Numerical analyses of AFLP patterns assigned *L. rhamnosus* PRSF-L477 to cluster VII, which was the largest cluster found in that study containing one commercial probiotic strain, two potentially probiotic strains, two food strains and forty-five human isolates, including thirty isolates from sterile sites and fifteen from commensal flora. PRSF-L477 grouped closely to *L. rhamnosus* LMG 6400^T = American Type Culture Collection (ATCC) 7469^T. Previously, two morphological mutants of *L. rhamnosus* ATCC 7469 were shown to exhibit increased platelet aggregation after gastrointestinal passage in gnotobiotic rats[43]. Platelet aggregation is a factor contributing to virulence and suggested to contribute to the progression of infective endocarditis[43]. In the present study, however, strain PRSF-L477 and one of its re-isolates were negative for platelet aggregation. In order to confirm that platelet aggregation of PRSF-L477 after passage through the gastrointestinal tract is not affected, more re-isolates should be included in this assay.

The viable count of *L. rhamnosus* increased in the faeces during supplementation with strain PRSF-L477, but it decreased rapidly after the end of the supplementation period. These findings suggest that PRSF-L477 does not colonise the gastrointestinal tract of healthy subjects and is washed out quickly after supplementation ends. This was also confirmed by the fact that PRSF-L477 re-isolates were not recovered during the 3-week wash-out period. Supplementation with *L. rhamnosus* PRSF-L477 did not appear to influence the total number of bacteria and the total number of anaerobic bacteria, but, as expected, it did influence the total number of lactobacilli. The number of lactobacilli increased during PRSF-L477 supplementation but decreased again when supplementation stopped. Studies on intestinal colonisation of *L. rhamnosus* reported similar results showing that the administered strains could easily be recovered during the intervention period but were washed out quickly during the post-administration period[24]. Re-isolates were only recovered from a few subjects after 1 week but were not detected after 2 weeks except for rare cases where re-isolates of the strain were detected after a few months.

Initial counts for lactobacilli were 1–2 $\log_{10}$ levels lower compared with plating results for lactobacilli in previous studies in human healthy volunteers[24,44], which might be due to differences in media composition or handling of faecal samples. The initial higher counts for the placebo group compared with the probiotic group for both lactobacilli and *L. rhamnosus* were not due to differences in baseline characteristics as these were equally divided over the two study groups. The small age difference between the two groups was non-significant (Mann–Whitney *U* test). The higher initial counts for both lactobacilli and *L. rhamnosus* in the placebo group were lost during the study. This loss was, however, non-significant with the exception of $t = 3$ weeks compared with $t = 0$ weeks for the lactobacilli in the placebo group ($P = 0{\cdot}015$; paired Wilcoxon test). As no intake of probiotic products was allowed during and 3 weeks before the study, a decrease in probiotic consumption in the placebo group was not likely to be the cause of this loss but cannot be excluded.

Conclusions

The results obtained in the course of a placebo-controlled double-blind volunteer trial indicate that the potentially probiotic strain *L. rhamnosus* PRSF-L477 is well tolerated and does not induce serious adverse events during or after administration. Therefore, it can be concluded that PRSF-L477 can safely be administrated to healthy adult subjects at a daily dose of 1×10^{11} cfu. Over the entire study period, nine re-isolates of this strain as confirmed by PFGE are phenotypically stable with regard to their antibiotic susceptibility profile. The strain does not appear to colonise the gastrointestinal tract of healthy subjects and is washed out quickly when supplementation ends. The present study thus provides the basis for further analysis of the efficacy of *L. rhamnosus* PRSF-L477 in a randomised double-blind placebo-controlled design according to FAO/WHO guidelines.

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R. D. W. initiated and coordinated the study. H. T. coordinated the volunteer trial as the clinical trial manager and was responsible for microbiological analysis. I. K. was responsible for antibiotic susceptibility profiling. G. H. was responsible for PFGE analysis and J. K. initiated the study and acted in an advisory role. R. D. W. wrote the manuscript. All authors read and contributed to the finalisation of the manuscript.

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