

# The Effect of a Multispecies Probiotic on the Intestinal Microbiota and Bowel Movements in Healthy Volunteers Taking the Antibiotic Amoxicillin

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- OBJECTIVE:** One of the side effects of antimicrobial therapy is a disturbance of the intestinal microbiota potentially resulting in antibiotic-associated diarrhea (AAD). In this placebo-controlled double-blind study, the effect of a multispecies probiotic on the composition and metabolic activity of the intestinal microbiota and bowel habits was studied in healthy volunteers taking amoxicillin.
- METHODS:** Forty-one healthy volunteers were given 500 mg amoxicillin twice daily for 7 days and were randomized to either 5 g of a multispecies probiotic, Ecologic® AAD (10<sup>9</sup> cfu/g), or placebo, twice daily for 14 days. Feces and questionnaires were collected on day 0, 7, 14, and 63. Feces was analyzed as to the composition of the intestinal microbiota, and  $\beta$ -glucosidase activity, endotoxin concentration, *Clostridium difficile* toxin A, short chain fatty acids (SCFAs), and pH were determined. Bowel movements were scored according to the Bristol stool form scale.
- RESULTS:** Mean number of enterococci increased significantly from log 4.1 at day 0 to log 5.8 (day 7) and log 6.9 (day 14) cfu/g feces ( $P < 0.05$ ) during probiotic intake. Although no other significant differences were observed between both intervention groups, within each group significant changes were found over time in both microbial composition and metabolic activity. Moreover, bowel movements with a frequency  $\geq 3$  per day for at least 2 days and/or a consistency  $\geq 5$  for at least 2 days were reported less frequently in the probiotic compared to the placebo group (48% vs 79%,  $P < 0.05$ ).
- CONCLUSION:** Apart from an increase in enterococci no significant differences in microbial composition and metabolic activity were observed in the probiotic compared with the placebo group. However, changes over time were present in both groups, which differed significantly between the probiotic and the placebo arm, suggesting that the amoxicillin effect was modulated by probiotic intake. Moreover, the intake of a multispecies probiotic significantly reduced diarrhea-like bowel movements in healthy volunteers receiving amoxicillin.

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## INTRODUCTION

One of the collateral effects of antimicrobial therapy is antibiotic-associated diarrhea (AAD), which can occur shortly after antibiotic intake up to 8 wk after cessation (1, 2). The incidence of AAD ranges from 5–39%, depending on the definition of diarrhea, the type of antibiotic used, and host factors (2). In general, amoxicillin, amoxicillin/clavulanic acid, clindamycin, and cephalosporines are associated with a high risk of AAD (3). AAD may range from mild disturbances to severe pseudomembranous colitis due to *Clostridium difficile* (4). This bacterium is thought to be the causative agent in up to 20% of AAD patients; however, the mechanisms causing the majority of cases of AAD are not clear (5, 6). Most of

the cases of AAD are thought to be due to a disturbance of the intestinal microbiota by antibiotics, which is associated with loss of colonization resistance (leading to overgrowth of potential pathogens), changes in carbohydrate digestion and production of short-chain fatty acids (SCFAs), altered metabolism of bile acids, and changes in both the mucosal and systemic immune response (7). In addition, antibiotics may have direct allergic and toxic effects on the mucosa, direct effects on immune-cell function, and pharmacological effects on intestinal motility (7–9). Possible consequences of AAD in health-care facilities include an increase in the incidence of nosocomial infections and an increase in morbidity and mortality, longer hospitalization, and higher costs of care (2). Although in general practice AAD is often merely

considered a nuisance, it may lead to a lack of compliance of antibiotic intake, which is associated with the development of antibiotic resistance (10). Furthermore, antibiotic use and the subsequent disturbance of the intestinal microbiota is a risk factor for the development of irritable bowel syndrome (11, 12).

Probiotics, which are defined as “mono- or mixed cultures of live microorganisms that, when applied to animal or human, beneficially affect the host by improving the properties of the indigenous microbiota,” may prevent and restore an imbalance caused by antibiotics and are therefore of increasing interest for the prevention and treatment of AAD (13). Several probiotic strains have been used in controlled studies, aiming at the prevention and treatment of AAD, such as *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* GG, *Bifidobacterium longum*, *Enterococcus faecium*, and *Saccharomyces boulardii*, and resulted in a significant decrease in the incidence of diarrhea (0–10% in the probiotic versus 14–27% in the placebo group) (14–22). However, other studies failed to show any benefit from probiotics in the prevention of AAD (23–25). Two meta-analyses on the use of probiotics in the prevention of AAD evaluated nine and seven placebo-controlled, double-blind trials, and reported an odds ratio of 0.37 and a relative risk of 0.40, respectively, in favor of probiotic administration (26, 27). A recent meta-analysis, which included 25 randomized controlled trials, confirmed these findings (relative risk of 0.43), and showed that the probiotic efficacy could be attributed to three types of probiotics: *S. boulardii*, *L. rhamnosus* GG, and probiotic mixtures (28). Most studies on probiotics and AAD have only investigated the development of diarrhea (*i.e.*, clinical outcome); only in a few studies the effect on the composition of the fecal microbiota was also examined, albeit to a very limited degree (21, 29, 30).

It has recently been demonstrated that multispecies and, to a lesser extent, multistrain probiotics have certain advantages over monostrain preparations. Mixed preparations may complement each other's effect through synergism and/or symbiosis (31). Ouweland *et al.* reported, for example, that the *in vitro* adhesion of *B. lactis* Bb12 was more than doubled by the presence of *L. rhamnosus* GG and *L. delbrueckii* spp. *Bulgaricus* (32). A progressive increase in *B. lactis* growth and acidification in the presence of *L. acidophilus in vitro* was demonstrated by Gomes *et al.* (33).

It is well known that the composition of the fecal microbiota and its role in colonization resistance, but also its metabolic activity (producing several bacterial enzymes, short chain fatty acids [SCFAs], amines, and bacteriocins), will affect the host. However, few studies have assessed the influence of probiotics on the metabolic activity of the intestinal microbiota during and after antibiotic treatment. Commonly, studies on AAD concerned the use of monospecies probiotics. The results of these studies are difficult to generalize, since there is a lot of variation between species; properties that apply to one strain are not necessarily applicable to another.

The objective of this placebo-controlled, randomized, double-blind study was to evaluate the effect of a multi-species probiotic on the composition of the intestinal microbiota in healthy volunteers during and after amoxicillin intake. Moreover, the effect of the probiotic on the metabolic activity of the intestinal microbiota and on bowel habits was studied.

## METHODS

### Subjects

Healthy volunteers between 18 and 65 yr of age were eligible for the study. Exclusion criteria were: smoking, pregnancy, lactation, hypersensitivity to  $\beta$ -lactam antibiotics or tetracycline, pre-existing bowel pathology (including irritable bowel syndrome, inflammatory bowel disease, diverticulitis, and cancer), treatment with immune-suppressive medication or immune-compromised subjects, diarrhea or constipation (in the last 3 days prior to inclusion), allergic and inflammatory reactions, as well as infections within 2 wk prior to inclusion. Furthermore, the volunteers were not allowed to use: (a) gastric acid inhibitors, laxatives, antidiarrhea medication, or antibiotics for at least 2 months before the start of and during the study; (b) corticosteroids for at least 4 wk before the start of and during the study; (c) other probiotics and prebiotics for at least 2 wk before the start of and during the study. Finally, participants were asked to continue their ordinary dietary habits. All volunteers gave written informed consent. The study was approved by the medical ethics committee of the University Hospital Maastricht, The Netherlands.

### Study Design

The study was executed according to a parallel, randomized, placebo-controlled, double-blind design. The total duration of the intervention and follow-up period was 63 days. Volunteers received 500 mg amoxicillin twice daily from day 1–7 and were randomized to receive either 5 g of a multi-species probiotic or 5 g placebo twice daily from day 1–14. This resulted in three time periods defined as: day 1–7, “the antibiotic/probiotic period,” in which all volunteers received amoxicillin in combination with either probiotic or placebo; day 8–14, “the probiotic only period,” in which volunteers received either probiotic or placebo; day 15–63, “the post-treatment follow-up period.” Amoxicillin was taken with milk before breakfast and dinner, while the placebo or probiotic was taken before lunch and before bedtime. The time between antibiotic and probiotic intake had to be at least 2 h. Fresh fecal samples were collected on day 0 (*i.e.*, baseline), 7, 14, and 63. On the same day, a questionnaire was filled out including questions on bowel movements (stool frequency and consistency [ranging from 1 = hard lumps to 7 = completely watery] according to the Bristol stool form scale (34)), use of pre- and probiotics, other medication taken, (drastic) change of eating habits, and compliance. In addition, a short questionnaire on bowel habits and side effects (nausea, abdominal

cramps, bloating, flatulence, or other) had to be completed daily during probiotic/placebo intake.

### Probiotic

The multispecies probiotic (Ecologic® AAD) and the placebo were kindly provided by Winclove Bio Industries, Amsterdam, The Netherlands. Ecologic® AAD consists of 10 different bacterial species at each  $10^8$  colony forming units (cfu)/g the total dose being  $10^9$  cfu/g (*B. bifidum* W23, *B. lactis* W18, *B. longum* W51, *E. faecium* W54, *L. acidophilus* W37 and W55, *L. paracasei* W72, *L. plantarum* W62, *L. rhamnosus* W71, and *L. salivarius* W24), 5% mineral mix (potassium chloride [~67.3%], magnesium sulphate [~32.6%] and manganese sulphate [~0.1%]) and 15% Raftilose® synergy1 (inulin enriched with oligofructose). Each participant consumed sachets containing 5 g Ecologic® AAD or placebo twice daily for 2 wk. Sachets were dissolved in lukewarm water, left for 10 min, stirred, and thereafter ingested. The placebo sachets were indistinguishable in color, smell, and taste from the probiotic sachets but contained no bacteria.

### Sample Processing

Fecal samples were brought to the laboratory within 12 h after defecation and divided into three portions: (a) ten grams was centrifuged at 47,000 g for 2 h at 4°C to obtain fecal water, which was frozen immediately in 2-fold at -80°C for analysis of endotoxin concentrations and determination of pH; (b) five grams was diluted (1:4) with peptone water (Oxoid CM9, Basingstoke, Hants, U.K.) supplemented with cysteine (2.1 mM) and glycerol (30%). Bacterial cultures of the fecal dilution were performed immediately and the remainder was frozen at -20°C for the subsequent analyses of enzyme activities, *Clostridium difficile* toxin A, and SCFAs, (c) the remaining fecal sample (1–15 grams) was frozen directly at -20°C for additional analyses.

### Bacteriological Culture

Tenfold serial dilutions of the fecal dilution were made in physiological saline (0.85%) with cysteine-HCl (0.05%) and 40 µL of these dilutions was inoculated using a spiral plater (Eddy Jet v1.2, IUL-instruments, Barcelona, Spain) onto the following agar plates: blood agar (Oxoid CM271) for total (facultative) aerobic bacteria, eosin-methylene blue (methylthioninium chloride) agar (Oxoid CM69) for enterobacteriaceae, KF-streptococcus agar (Oxoid CM701) for enterococci, fastidious anaerobic agar (Laboratory M LabGo, Lancashire, U.K.) for total (facultative) anaerobic bacteria, bile-esculine agar (Becton Dickinson 287920, La Pont de Claix, France) for *Bacteriodes spp.*, LAMVAB agar for lactobacilli, Sabouraud agar with gentamicin and chloramphenicol (GM+C) (Becton Dickinson 254041) for yeasts and egg yolk-neomycin agar for spore-forming clostridia. LAMVAB agar was prepared according to the method described by Hartemink *et al.* (35). Egg yolk-neomycin agar was prepared by adding a sterile neomycin solution (final concentration, 100 µg/mL) to egg yolk agar with freshly prepared egg yolk

emulsion (36). Before inoculation of the egg yolk-neomycin agar, fecal dilutions were heated at 80°C for 10 min.

Blood agar and eosin-methylene blue agar plates were incubated aerobically at 37°C for 24 h. Sabouraud GM+C and KF-streptococcus agar plates were incubated aerobically at 37°C and 42°C, respectively, for 48 h. Fastidious anaerobic, bile-esculine, egg yolk, and LAMVAB agar plates were incubated under anaerobic conditions at 37°C for 48 h.

### Viability of *E. faecium* W54

From each KF-streptococcus agar plate two dominant colonies were isolated on both day 7 and 14 and purified on blood agar plates. Isolates were frozen at -80°C awaiting further identification. At the end of the study, the enterococci of 10 individuals (*i.e.*, 40 colonies), who had received probiotic treatment, were typed by pulse field gel electrophoreses (PFGE), using *SmaI* according to the method described by van den Braak *et al.* (37, 38).

### Bacterial Enzyme Activity

Bacterial β-glucosidase activity was determined as previously described (39). Briefly, fecal dilutions were mixed (1:1) with 0.1 M PBS (pH 6.8), sonicated for 1 min, and centrifuged at 1,700 g for 15 min. The supernatants were lyophilized for 75 min by Speed-Vac (Savant DNA 120, GMI, Inc., Ramsey, MN) and the remaining fractions were used to determine β-glucosidase (at 420 nm) activity by using p-nitrophenyl β-D-glucopyranoside as a substrate.

### Endotoxin

The endotoxin (*i.e.*, lipopolysaccharide) concentration was determined in fecal water using the *Limulus* amoebocyte lysate endochrome technique (Endosafe, end point chromogenic analysis endochrome test kit, Charles River, Kent, U.K.). The analysis was performed according to the manufacturer's specifications under pyrogen-free conditions. Pyrogen-free water was used to dilute the fecal samples, and the test-solutions and as negative control. The detection range of the assay was 0.015 to 0.12 EU/mL (9 EU/ng). Concentration of fecal endotoxin was expressed as nanogram of endotoxin per mL of fecal water.

### *Clostridium difficile* Toxin A

*Clostridium difficile* toxin A was determined using an enzyme-linked fluorescent immunoassay technique (VIDAS® *C. difficile* Toxin A II assay, bioMérieux, Lyon, France). The analysis was performed according to the manufacturer's specifications using the VIDAS system (bioMérieux, Lyon, France).

### Short-Chain Fatty Acids

SCFAs were measured in the fecal dilutions using gas-liquid chromatography. The gas-liquid chromatography system consisted of a CP9002 gas chromatograph equipped with a flame ionization detector in conjunction with Maestro software (Chrompack, Middelburg, The Netherlands) for

calculations. The chromatographic column used was WCOT fused silica (25 m × 0.32 mm id), coated with FFAP-CB df 0.3. This column was used in an isothermal mode at 140°C and both the injector and detector temperature were 270°C. The sample size was 1.0 μL, which was split 50:1 to give a 0.02-μL sample on the column. Helium was used as the carrier gas with a head pressure of 0.8 bar. SCFAs were extracted and analyzed as previously described (39).

### pH

The pH of fecal water was determined using a PHM standard pH meter with a PHC3006 electrode (Radiometer Nederland BV, Zoetermeer, The Netherlands).

### Protein Concentration

Homogenized fecal samples were diluted (1:99) in 0.1 M PBS (pH 6.8, 5–7°C) and added to BioRad Assay Protein Dye Reagent (1:1). After 30 min, the absorbance was read at 595 nm. Concentrations of proteins were calculated from a standard curve for proteins ranging from 0–120 μg/mL and expressed as mg total protein per gram feces.

### Defecation Score

In this study a diarrhea-like defecation has been defined as a defecation frequency ≥3 per day and/or a fecal consistency ≥5 per day, on the Bristol stool form scale, for at least 2 days.

### Statistics

The treatment allocation was concealed to all investigators and volunteers, until the study had been completed and all analyses had been performed.

The primary outcome of this study was to compare the changes that occurred in the composition of the intestinal microbiota during and after amoxicillin intake between probiotic- and placebo-treated subjects. Secondary outcomes were the changes that occurred in the metabolic activity of the intestinal microbiota and changes in defecation score during and after amoxicillin between probiotic- and placebo-treated subjects.

Only data from volunteers who completed the study, had a probiotic/placebo and antibiotic compliance of ≥90%, and delivered all four fecal samples were included in the data analyses.

Statistical evaluation of differences between groups and changes within groups (at all time points during the study period) was carried out using linear mixed model analysis. In this analysis the fixed effects were day and treatment and the random effect was subject. For two-group comparisons of independent ordinal and interval values the nonparametric Mann-Whitney U-test was used while the nonparametric Wilcoxon signed-ranked test was used for comparison of related ordinal and interval values. All tests were conducted using SPSS version 11.0 (SPSS Inc, Chicago, IL) and a *P* value below 0.05 was considered statistically significant.

Based on data from previous probiotic studies, it was estimated that 19 volunteers per treatment group would provide 80% power to detect a one log difference in numbers of specific microorganisms cultured, assuming a variance of 1.1 and a 2-sided significance level of 0.05.

## RESULTS

### Subjects

Forty healthy volunteers completed the study, 19 in the probiotic (5 men and 14 women, mean age 25.5, SD 10.2 yr) and 21 in the placebo group (10 men and 11 women, mean age 28.2, SD 11.5 yr). One subject in the probiotic group was found to be allergic to amoxicillin and had to be excluded. Two subjects in the placebo group did not complete the daily questionnaire. On day 14, subjects delivered fecal samples to the hospital and subsequently handed in the daily questionnaire. As a consequence information from the questionnaire was available for day 1–13. The compliance for antibiotic intake was at least 93%, and for probiotic/placebo intake at least 97% in both groups. One subject in the placebo group and three in the probiotic group incidentally (*i.e.*, maximally twice a week) consumed yogurt containing *L. rhamnosus* GG between day 14 and 7 before starting the study. Moreover, in the probiotic group one other subject incidentally consumed that probiotic during the first 2 wk of the study. Apart from one subject in the probiotic group taking omeprazole once daily on day 45 and 46 of the study, no medication potentially affecting the intestinal microbiota was taken during the study.

### Bacteriological Culture

During probiotic intake, a significant increase in the mean number of fecal enterococci was found on day 7 (5.8 vs 4.0 log cfu/g feces, *P* < 0.02) and on day 14 (6.9 vs 4.3, *P* < 0.001) in the probiotic group compared to the placebo group (Table 1). Moreover, the mean number of fecal enterococci within the probiotic group increased significantly during antibiotic/probiotic intake (day 7) and increased further during probiotic therapy alone (day 14). A significant decrease in the mean number of fecal enterococci was observed 7 wk after cessation of probiotic intake (*P* < 0.05) having returned to pretreatment level (Table 1). No further differences in either aerobic or anaerobic bacterial species could be seen between the probiotic and the placebo group.

However, group-specific differences were observed over time: within the probiotic group a significant decrease was found in total aerobes (day 63 versus day 7) and significant increases were observed over time in total anaerobes (day 14 versus day 0) and *Bacteroides* spp. (day 7 and day 14 versus day 0) (*P* < 0.05). Within the placebo group a significant increase was found over time in enterococci (day 14 versus day 0) and significant decreases were found in lactobacilli (day 7 versus 0) and spore-forming clostridia (day 7 versus day 0 and 63) (*P* < 0.05) (Table 1).

**Table 1.** Numbers of Bacteria Cultured Expressed as Log cfu/g Feces

	Day 0 Mean (± SEM)	Day 7 Mean (± SEM)	Day 14 Mean (± SEM)	Day 63 Mean (± SEM)
Total aerobic microbiota				
Probiotic <sup>†</sup>	7.0 (0.2)	7.3 (0.1)	6.9 (0.2)	6.8 (0.2)
Placebo	6.8 (0.1)	7.1 (0.2)	6.8 (0.2)	7.1 (0.2)
Enterobacteriaceae				
Probiotic	6.1 (0.3)	6.5 (0.3)	5.9 (0.3)	6.0 (0.3)
Placebo	6.6 (0.2)	6.6 (0.2)	6.3 (0.2)	6.4 (0.4)
Enterococci				
Probiotic <sup>‡</sup>	4.1 (0.3)	5.8 (0.3)*	6.9 (0.3)*	4.4 (0.3)
Placebo <sup>§</sup>	3.5 (0.3)	4.0 (0.3)	4.3 (0.3)	4.2 (0.4)
Total anaerobic microbiota				
Probiotic <sup>¶</sup>	8.2 (0.2)	8.3 (0.2)	8.7 (0.2)	8.7 (0.2)
Placebo	8.0 (0.2)	8.2 (0.2)	8.1 (0.3)	8.5 (0.2)
<i>Bacteroides spp.</i>				
Probiotic <sup>  </sup>	6.1 (0.2)	6.8 (0.1)	6.6 (0.2)	6.5 (0.1)
Placebo	6.1 (0.1)	6.5 (0.2)	6.2 (0.1)	6.4 (0.2)
Spore-forming clostridia				
Probiotic	4.6 (0.2)	4.2 (0.2)	4.2 (0.3)	4.3 (0.2)
Placebo**	4.5 (0.2)	3.8 (0.2)	4.2 (0.3)	4.5 (0.2)
Lactobacilli				
Probiotic	4.7 (0.3)	4.7 (0.2)	5.0 (0.3)	4.6 (0.4)
Placebo <sup>††</sup>	5.1 (0.3)	4.3 (0.3)	4.6 (0.4)	4.7 (0.4)

\*Between group difference  $P < 0.02$ .

<sup>†</sup>within group decrease  $t = 63$  versus  $t = 7$ ,  $P < 0.05$ .

<sup>‡</sup>within group increase  $t = 7/14$  versus  $t = 0/63$  and  $t = 7$  versus  $t = 14$ ,  $P < 0.05$ .

<sup>§</sup>within group increase  $t = 14$  versus  $t = 0$ ,  $P < 0.05$ .

<sup>¶</sup>within group increase  $t = 14$  versus  $t = 0$ ,  $P < 0.05$ .

<sup>||</sup>within group increase  $t = 7/14$  versus  $t = 0$ ,  $P < 0.05$ .

\*\*within group decrease  $t = 7$  versus  $t = 0/63$ ,  $P < 0.05$ .

<sup>††</sup>within group decrease  $t = 7$  versus  $t = 0$ ,  $P < 0.05$ .

For all bacterial species studied in both the probiotic and the placebo group, values on day 63 did not differ significantly from day 0.

The PFGE profile of 39 out of the 40 enterococci strains, isolated from the feces of the healthy volunteers receiving probiotic, was similar to that of the orally administered probiotic *E. faecium* W54 strain.

**Metabolic Activity**

$\beta$ -Glucosidase activity did not differ significantly between the probiotic and the placebo group during the total study period (Table 2). Within both groups, a decrease in  $\beta$ -glucosidase was observed at day 7 (significant for the placebo group), which increased again on day 63 (significant for the probiotic group). In both groups, the  $\beta$ -glucosidase activity returned

**Table 2.** Metabolic Activity:  $\beta$ -Glucosidase Activity (Expressed as mg/60 min/g Feces); SCFA Concentration (Expressed in mmol/g Feces) and pH

		Day 0 Mean (± SEM)	Day 7 Mean (± SEM)	Day 14 Mean (± SEM)	Day 63 Mean (± SEM)
$\beta$ -glucosidase	Probiotic*	0.87 (0.19)	0.57 (0.14)	0.58 (0.14)	0.88 (0.19)
	Placebo <sup>†</sup>	0.75 (0.14)	0.40 (0.11)	0.60 (0.14)	0.67 (0.16)
SCFA					
Acetic acid	Probiotic <sup>‡</sup>	110.6 (12.0)	83.9 (11.2)	84.1 (11.6)	92.3 (11.2)
	Placebo <sup>§</sup>	103.6 (15.6)	78.8 (14.9)	105.3 (17.1)	79.3 (12.5)
Propionic acid	Probiotic <sup>¶</sup>	29.4 (3.6)	35.9 (3.9)	24.6 (3.5)	26.1 (3.1)
	Placebo	28.5 (5.6)	26.5 (5.8)	25.6 (3.1)	24.1 (4.6)
Butyric acid	Probiotic <sup>  </sup>	30.9 (5.1)	19.2 (3.7)	19.3 (3.0)	30.2 (5.6)
	Placebo**	30.1 (6.1)	13.4 (2.7)	22.4 (4.0)	19.1 (3.9)
pH	Probiotic	6.5 (0.2)	6.3 (0.2)	6.5 (0.2)	6.7 (0.2)
	Placebo	6.4 (0.1)	6.6 (0.2)	6.5 (0.2)	6.5 (0.2)

\*within group increase  $t = 63$  versus  $t = 7$ ,  $P < 0.05$  and a tendency to a within group decrease  $t = 7$  versus  $t = 0$ , and increase  $t = 63$  versus  $t = 14$ ,  $P < 0.06$ .

<sup>†</sup>within group decrease  $t = 7$  versus  $t = 0$ ,  $P < 0.05$ .

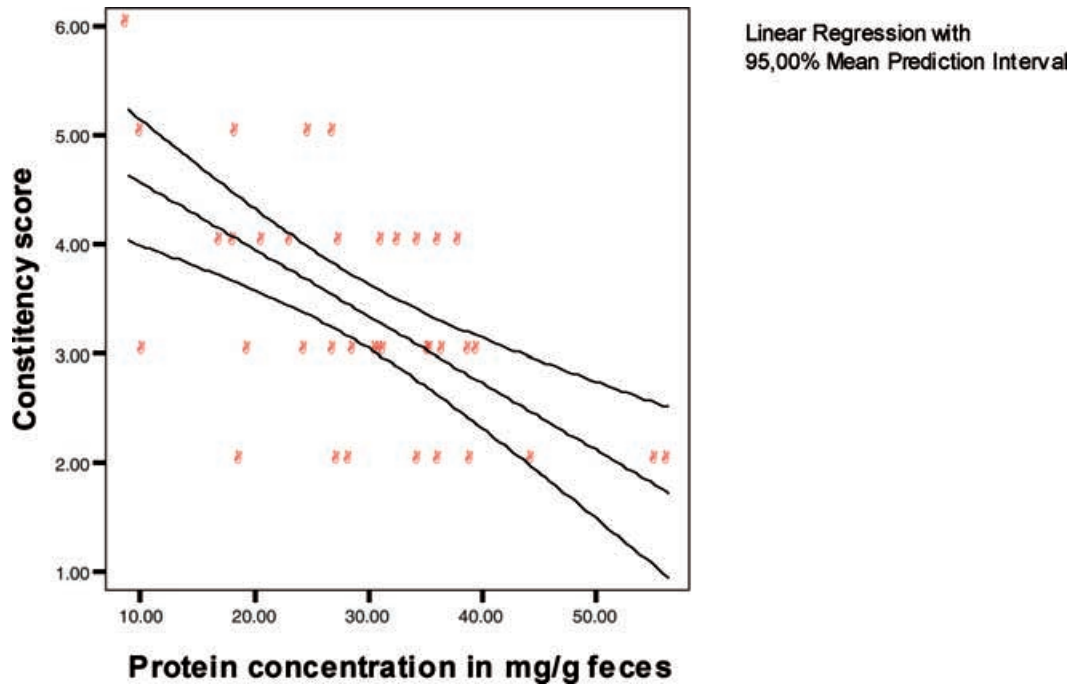
<sup>‡</sup>within group decrease  $t = 7/14$  versus  $t = 0$ ,  $P < 0.05$ .

<sup>§</sup>within group increase  $t = 14$  versus  $t = 7$ ,  $P < 0.05$  and within group decrease  $t = 63$  versus  $t = 14$ ,  $P < 0.05$ .

<sup>¶</sup>within group decrease  $t = 14/63$  versus  $t = 7$ .

<sup>||</sup>within group decrease  $t = 7/14$  versus  $t = 0$ ,  $P < 0.05$  and within group increase  $t = 63$  versus  $t = 7/14$ ,  $P < 0.05$ .

\*\*within group decrease  $t = 7/63$  versus  $t = 0$ ,  $P < 0.05$ .



**Figure 1.** Linear regression of fecal consistency (scored with the Bristol stool form scale) in relation to the protein concentration per gram feces ( $R = 0.61$ ,  $P < 0.01$ ). Consistency ranging from 1 (hard lumps) to 7 (watery) according to the Bristol stool form scale. Red dots represent fecal samples from each volunteer collected on day 14.

to baseline values 7 wk after cessation of amoxicillin intake (day 63).

Endotoxin concentrations (mean  $\pm$  SEM), expressed as log ng/mL fecal water, did not differ significantly between the probiotic and the placebo group on day 0 ( $2.15 \pm 0.07$  vs  $2.04 \pm 0.08$ ), day 7 ( $2.30 \pm 0.06$  vs  $2.22 \pm 0.07$ ), and day 14 ( $1.92 \pm 0.10$  vs  $1.89 \pm 0.11$ ). However, in both groups, a small but not significant increase in mean endotoxin concentration was observed on day 7, whereas 1 wk after cessation of antibiotic intake (day 14) a significant decrease ( $P < 0.05$ ) in endotoxin concentration was observed compared to day 7.

*Clostridium* toxin A was detected in the feces of two volunteers in the placebo group 1 wk after cessation of antibiotic intake (day 14) and in one volunteer in the probiotic group at the start of the study (day 0).

No significant differences between the groups were observed for any of the SCFAs tested (Table 2). However, within both groups changes were found over time. Butyric acid concentrations significantly decreased in both groups during antibiotic intake, but by day 63 had recovered to baseline in

the probiotic group. This effect was also observed for acetic acid. Furthermore, an increase in propionic acid concentrations was observed on day 7 in the probiotic group.

No significant changes were found in the pH of the fecal water between and within both groups, during the total study period (Table 2).

At all time points, a negative correlation ( $P < 0.05$ ) was observed between the amount of protein per gram feces and the consistency score (except at  $t = 63$  days). In addition, a positive correlation ( $P < 0.05$ ) was observed between the amount of fecal water per 10 g of feces and the consistency score at all time points (an example is shown in Fig. 1).

#### Defecation Score

The mean defecation frequency and consistency before antibiotic and probiotic intake (day 0, *i.e.*, baseline), during the antibiotic/probiotic period (day 1–7), and during the probiotic only period (day 8–13) are listed in Table 3. The defecation frequency during the probiotic only period (day 8–13) was significantly lower ( $P < 0.05$ ) in the probiotic than in the

**Table 3.** Mean Daily Fecal Frequency and Consistency Scores Before Antibiotic/Probiotic Intake (Day 0), During Antibiotic/Probiotic Intake (Day 1–7), and During Probiotic Intake Alone (Day 8–13)

	Frequency Mean (SEM)			Consistency* Mean (SEM)		
	Day 0	Day 1–7	Day 8–13	Day 0	Day 1–7	Day 8–13
Probiotic (N = 19)	1.0 (0.2)	1.4 (0.1)	1.2 (0.1) <sup>†</sup>	3.5 (0.3)	3.9 (0.1)	3.5 (0.1)
Placebo (N = 19)	0.9 (0.2)	1.5 (0.1)	1.5 (0.1)	4.0 (0.3)	3.9 (0.1)	3.8 (0.1)

\*Consistency ranging from 1 (hard lumps) to 7 (watery) according to the Bristol stool form scale.

<sup>†</sup>Significant difference between probiotic and placebo group ( $P < 0.05$ ).

**Table 4.** Defecation Score Between Day 1 and Day 13

	Placebo N = 19	Probiotic N = 19
1. Stool frequency $\geq 3$ per day for at least 2 days	11%	11%
2. Stool consistency <sup>1*</sup> $\geq 5$ for at least 2 days	42%	21%
3. Stool frequency $\geq 3$ per day and a consistency <sup>1</sup> $\geq 5$ for at least 2 days	26%	16%
4. Stool frequency $\geq 3$ per day and/or a consistency $\geq 5$ for at least 2 days (being the sum of 1–3)	79%*	48%

\* $P < 0.05$ .<sup>1</sup>Consistency ranging from 1 (hard lumps) to 7 (watery) according to the Bristol stool form scale.

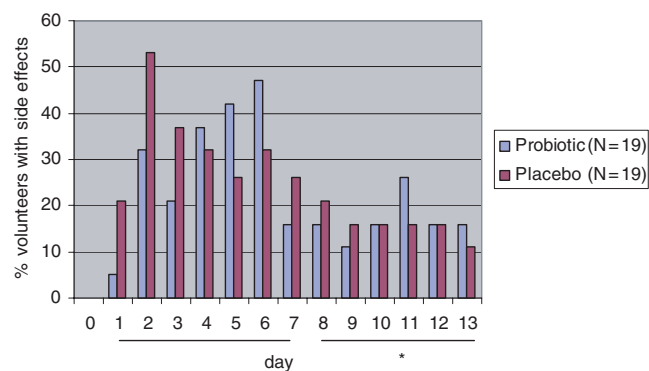
placebo group. During the total probiotic period (day 1–14), diarrhea-like bowel movements were reported less frequently in the probiotic (48%) than in the placebo (79%) group ( $P < 0.05$ ) (Table 4).

### Side Effects

The placebo and probiotic group were comparable regarding the percentage (Fig. 2) and severity of side effects reported: 79% mild-moderate symptoms in the probiotic group *versus* 90% mild-moderate symptoms in the placebo group. Side effects most frequently reported were nausea, abdominal cramps, bloating, and flatulence. Finally, side effects were significantly more frequent during the antibiotic/probiotic period (day 1–7) than during the probiotic only period (day 8–13) ( $P < 0.05$ ), for both the probiotic and placebo group (Fig. 2).

## DISCUSSION

In this placebo-controlled double-blind study, investigating the effect of a multispecies probiotic in healthy volunteers after amoxicillin intake, no differences in the composition of the intestinal microbiota were observed in the probiotic group



**Figure 2.** Side effects. \*Significant difference ( $P < 0.05$ ) in the percentage of volunteers with side effects during day 1–7 *versus* day 8–13.

*versus* the placebo group, apart from a significant increase in fecal enterococci. Although no other differences were observed between groups, group-specific changes were seen over time. Such changes were also observed for metabolic activity. Finally, a significantly better defecation score (decrease in diarrhea-like bowel movements) was observed in the probiotic group *versus* the placebo group.

In AAD, differences in efficacy have been reported for different bacterial species, bacterial strains, and probiotic mixtures (28). The efficacy of multispecies probiotic mixtures is further supported by the successful use of the multispecies probiotic VSL#3 in several gastrointestinal disorders (40–42). In the present study, we used a multispecies probiotic containing 10 different probiotic strains selected on the basis of their *in vitro* ability to inhibit growth of *Clostridium* spp. and to survive a low pH (2.5) as well as bile and digestive enzymes (pancreatin and pepsin) (data not shown). In addition, their resistance profile against a wide range of antibiotics was taken into account to prevent possible transfer of resistance from the probiotic bacteria to the indigenous microbiota. Finally, the combination of strains chosen was tested to exclude antagonistic effects in growth.

The composition of the fecal microbiota regarding total aerobic bacteria, clostridia, and lactobacilli counts before intervention was comparable with previous findings in healthy volunteers using the same culture methods (39, 43). However, in this study lower mean fecal bacterial concentrations were found for total anaerobic bacteria and *Bacteroides* spp., which cannot be explained.

One of the possible mechanisms by which probiotics exert their effect is by affecting the composition of the intestinal microbiota and preventing the overgrowth of possible pathogens. Only the fecal microbiota was investigated in this study, even though mucosa-associated bacteria may also be very relevant. Due to interindividual variation and possible sampling error, various biopsies ought to have been taken at all the different time points of the study. Considering the invasiveness and the potential risks this was considered not to be ethically acceptable in healthy volunteers.

The consumption of the multispecies probiotic, containing *E. faecium*, was associated with a significant increase in the concentration of fecal enterococci in the probiotic group from log 4.1 cfu/g to log 5.8 cfu/g on day 7 and to log 6.9 cfu/g on day 14. This increase disappeared 7 wk after cessation of probiotic intake, demonstrating that the consumption of this multispecies probiotic, containing *E. faecium*, can transiently alter the number of viable enterococci. Considering a consumption of  $1 \times 10^9$  cfu *E. faecium* per day, present in the multispecies product, and a fecal volume of 100 g per day, the recovery of around log 7 enterococci per gram feces after probiotic intake indicates that *E. faecium* is able to survive passage through the gastrointestinal tract very well. Moreover, the PFGE patterns of the enterococci isolated from the fecal samples were similar to the orally administered *E. faecium*. A study in which a monospecies *E. faecium* probiotic ( $4.5\text{--}7.5 \times 10^9$  cfu daily) was given to healthy volunteers also

found a high increase (100-fold) in the total number of enterococci (44). Furthermore, the recovery of viable enterococci in the placebo group was not affected during amoxicillin intake, indicating that in this study amoxicillin had little effect on the indigenous enterococci population.

In contrast to the counts of enterococci, twice daily probiotic consumption containing  $3 \times 10^9$  cfu lactobacilli (*L. salivarius*, *L. plantarum*, and *L. rhamnosus*) did not significantly increase the number of fecal lactobacilli. However, a previous study by our group, in which 20 healthy volunteers consumed *L. plantarum* 299v for 4 wk did show a 1000-fold increase in the mean number of fecal lactobacilli (39). An increase in the mean fecal number of lactobacilli was also observed by others after a 6-month consumption of *L. rhamnosus* and a 3-wk consumption of *L. acidophilus* (45, 46). However, during amoxicillin intake a decrease was observed in the total number of fecal lactobacilli in the placebo group but not in the probiotic group. Comparable results were observed in a study performed by Plummer *et al.* in which probiotic supplementation was given during *H. pylori* eradication therapy (30). These findings indicate that probiotic intake might prevent a decrease of lactobacilli caused by antibiotic intake.

Apart from lactobacilli and the *E. faecium*, the multispecies probiotic used also contained bifidobacteria. However, no bifidobacteria were cultured, due to insufficient selectivity and sensitivity of media available. In future studies, quantification of bifidobacteria ought to be performed with molecular-based techniques.

Looking at both intervention groups, specific changes during and after amoxicillin intake were observed, indicating an effect of amoxicillin intake on the gut microbiota. These results are in line with the literature (47–50). The various effects over time in the probiotic group compared to the placebo group suggest that the intake of the multispecies probiotic had an impact on the microbiota during amoxicillin intake, possibly contributing to the better defecation score. This probiotic effect on the microbiota is partly caused by the bacteria themselves, as is reflected in the increase of enterococci in the probiotic group. In addition, the increase in, for example, the total anaerobic microbiota and the absence of a decrease in the spore-forming clostridia during amoxicillin intake suggests that the probiotic bacteria were able to induce a change in the intestinal environment favoring the growth of these commensal organisms. The fact that the differences between the groups were not significant is probably due to the high interindividual variation.

Alteration of the colonic microbiota due to antibiotic treatment can result in overgrowth of *C. difficile* in the colon. However, no increase in *Clostridium* spp. was observed in either group during or after antibiotic therapy. Moreover, during antibiotic therapy *Clostridium* toxin A was not detected in the stool of any of the volunteers. This was to be expected as the prevalence of *C. difficile* colonization among healthy adults is very low, generally less than 2% (51). The spores of these bacteria are usually acquired from hospitals and long-term-care facilities where the prevalence ranges from

5–20% (52, 53), which can further increase with length of stay (54).

A change in the composition of the intestinal microbiota might affect its metabolic characteristics, such as  $\beta$ -glucosidase activity.  $\beta$ -Glucosidase has been implicated in carcinogenesis, since it is able to hydrolyse dietary substrates into carcinogenic compounds (55, 56). A decrease of this activity is therefore potentially beneficial. It has been demonstrated that a change in the composition of the intestinal microbiota or the intake of *Lactobacillus* spp. can influence  $\beta$ -glucosidase activity, although the results differ between strains and populations studied (39, 46, 57–61). In the present study  $\beta$ -glucosidase activity decreased in both groups during amoxicillin intake and returned to baseline values 7 wk after cessation of amoxicillin. No effect of probiotic intake was observed.

The endotoxin concentrations in both groups increased during amoxicillin intake, though not significantly, and decreased significantly 1 wk after cessation of amoxicillin intake. This is in accordance with evidence from several studies showing that antibiotics increase the bioavailability of endotoxin originating from Gram-negative bacteria (62–65). The level of intestinal endotoxin, however, does not only correlate with the number of Gram-negative bacteria, which is in line with the fact that no changes were seen in total counts of enterobacteriaceae, but can also be associated with the metabolic activity associated with proliferation (62). The clinical significance of antibiotic-induced endotoxin release remains to be clarified. There is evidence that endotoxemia may be of importance in patients with increased gut permeability and that probiotics show potential in preventing loss of gut barrier integrity (66, 67). Some studies suggest that a reduction in intestinal endotoxin concentration may be associated with decreased endotoxin leakage across the gut wall, and subsequently with the control of endotoxin-related conditions (68). In our study, probiotic intake had no effect on intestinal endotoxin concentrations.

The major SCFAs arising from the bacterial fermentation of nondigestible carbohydrates are acetic acid, propionic acid, and butyric acid. They serve as important energy sources (mostly butyric acid) for colonocytes, are associated with the regulation of water and electrolyte transport, and decrease colonic pH, thereby inhibiting overgrowth of potential pathogens (69). In a study with 31 severe AAD patients disturbances in the intestinal microbiota were observed as a reduction of the amounts of all major fecal SCFAs (70). SCFA concentrations and anaerobic cultural counts also decreased after systemic ceftriaxone treatment in 10 healthy volunteers (71). Probiotics, by interacting with the intestinal microbiota and being saccharolytic, can alter SCFA concentrations in the colon. Studies have demonstrated different effects on SCFA concentrations after probiotic intake, with some showing no effect (39, 43, 72–74), and others showing either an increase (75, 76) or a decrease in specific SCFA concentrations (61). Possible explanations for these inconsistent findings are the techniques applied, the populations studied, and the different

probiotic strains used. In the present study, decreased acetic acid and butyric acid concentrations were observed during antibiotic treatment, only returning to baseline 7 wk after cessation of antibiotic intake in the probiotic group. Moreover, an increased propionic acid concentration was observed in the probiotic group at day 7. In contrast, the main fermentation products of the bacteria present in the multispecies probiotic are lactate, acetate, and formate (the latter only formed by bifidobacteria) and do not include propionate. In this respect, metabolic cross-feeding is likely to have occurred as lactate can be converted into butyrate or propionate. Which metabolic pathway is utilized depends on the composition of the microbiota as well as environmental conditions, and shows high interindividual variation (77, 78). In general, the overall SCFA concentration seemed to be higher in the probiotic group, which could be one of the explanations for the less diarrhea-like defecation score in this group, due to a better water and electrolyte absorption (79). It should be noted that only 1–5% of the amount of SCFAs produced is excreted in the feces and that changes in SCFA concentration can be due to both changes in production and/or absorption and altered motility (80).

This study demonstrated that the intake of a multispecies probiotic resulted in a significantly better defecation score (decrease in diarrhea-like bowel movements), which is in accordance with previous studies showing that probiotics significantly reduce the relative risk of developing AAD (26–28). Fecal consistency was estimated by the validated Bristol stool form scale. The significant correlation of the consistency with both the amount of protein and fecal water per gram feces supports the validity of this scale.

During antibiotic intake a significant number of side effects was reported in both groups, but their numbers did not differ between the probiotic and the placebo group. These results suggest that the multispecies probiotic that was able to decrease diarrhea-like defecation does not reduce other gastrointestinal side effects, but also does not result in adverse events. The clinical relevance of the improved defecation score has to be further studied in specific patient populations who have an increased risk of AAD due to host factors (age, immune status), hospitalization status, and exposure to higher doses of antibiotics (2).

The compliance rates for both antibiotic and probiotic/placebo intake were high in this study, although we readily admit that this was self-reported. We also acknowledge that many gastrointestinal bacteria remain uncultured and that molecular-based techniques would allow a more complete assessment of microbial diversity. However, culture provides information on quantitative alterations in viable counts of specific groups of bacteria, which are also important for the metabolic activity of the intestinal microbiota.

In conclusion, comparing the specially developed multispecies probiotic Ecologic<sup>®</sup> AAD with placebo no differences were observed in bacterial counts nor in metabolic activity, apart from an increase in enterococci. However, changes over time were present in both groups indicating an

amoxicillin effect, which differed between the probiotic and the placebo group. Moreover, the intake of a multispecies probiotic significantly reduced diarrhea-like bowel movements in healthy volunteers receiving amoxicillin. Although the changes over time in microbial composition and metabolic activity by themselves were small, the sum of potentially beneficial changes may have contributed to the improved defecation score observed. The present study therefore supports the hypothesis that multispecies probiotics could be used in the prevention of AAD, as they affect the composition and function of the intestinal microbiota.

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## STUDY HIGHLIGHTS

### What Is Current Knowledge

- The disruption of the intestinal microbiota by antibiotics may result in antibiotic-associated diarrhea (AAD).
- Clinical studies show that probiotics seem efficacious in preventing AAD.
- Beneficial probiotic effects differ per probiotic bacterial strain used.

### What Is New Here

- Amoxicillin intake affects both the microbial composition and metabolic aspects of the fecal microbiota.
- The multispecies probiotic Ecologic<sup>®</sup> AAD affects both the composition as well as the metabolic activity of the fecal microbiota in healthy volunteers taking amoxicillin.
- The multispecies probiotic Ecologic<sup>®</sup> AAD causes a small but significant reduction in diarrhea-like bowel movements in healthy volunteers taking amoxicillin.

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#### CONFLICT OF INTEREST

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M.A.E. Jonkers were responsible for the analyses of the study. All authors participated in the design and data interpretation of the study and contributed significantly to the various drafts of the manuscript.

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