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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

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The probiotic potential of 47 selected strains of *Lactobacillus* spp. was investigated. The strains were examined for resistance to pH 2.5 and 0.3% oxgall, adhesion to Caco-2 cells, and antimicrobial activities against enteric pathogenic bacteria in model systems. From the results obtained in vitro, five strains, *Lactobacillus rhamnosus* 19070-2, *L. reuteri* DSM 12246, *L. rhamnosus* LGG, *L. delbrueckii* subsp. *lactis* CHCC 2329, and *L. casei* subsp. *alactus* CHCC 3137, were selected for in vivo studies. The daily consumption by 12 healthy volunteers of two doses of 10^{10} freeze-dried bacteria of the selected strains for 18 days was followed by a washout period of 17 days. Fecal samples were taken at days 0 and 18 and during the washout period at days 5 and 11. *Lactobacillus* isolates were initially identified by API 50CHL and internal transcribed spacer PCR, and their identities were confirmed by restriction enzyme analysis in combination with pulsed-field gel electrophoresis. Among the tested strains, *L. rhamnosus* 19070-2, *L. reuteri* DSM 12246, and *L. rhamnosus* LGG were identified most frequently in fecal samples; they were found in 10, 8, and 7 of the 12 samples tested during the intervention period, respectively, whereas reisolations were less frequent in the washout period. The bacteria were reisolated in concentrations from 10^5 to 10^8 cells/g of feces. Survival and reisolation of the bacteria in vivo appeared to be linked to pH tolerance, adhesion, and antimicrobial properties in vitro.

It is well known that the presence of lactobacilli is important for the maintenance of the intestinal microbial ecosystem (39). They have been shown to possess inhibitory activity toward the growth of pathogenic bacteria such as *Listeria monocytogenes* (3, 25, 36, 42), *Escherichia coli*, *Salmonella* spp. (8, 16, 27), and others (4, 13, 37). This inhibition could be due to the production of inhibitory compounds such as organic acids, hydrogen peroxide, bacteriocins (30), or reuterin (4) or to competitive adhesion to the epithelium. In order to survive in and colonize the gastrointestinal tract, probiotic bacteria should express high tolerance to acid and bile and have the ability to adhere to intestinal surfaces (31, 34). Survival in and temporary colonization of the human gastrointestinal tract have been demonstrated for some lactic acid bacteria (1, 23, 29). However, in vivo testing is expensive and time consuming and requires approval by ethical committees. Therefore, reliable in vitro methods for selection of promising strains are required.

Enterocyte-like Caco-2 cells (38) have been successfully used for in vitro studies on the mechanism of cellular adhesion of nonpathogenic lactobacilli (10, 24, 40, 43) and bifidobacteria (5, 15). Moreover, this cell line has been used to examine the mechanism of cellular adhesion and invasion of pathogenic bacteria such as *L. monocytogenes* (21), *Salmonella typhimurium* (20), and *E. coli* (32). Recently, Caco-2 cells have been used to examine the antimicrobial activity of lactobacilli (6, 13, 27) and bifidobacteria (5) against pathogenic bacteria. Antimicrobial properties of lactobacilli have been determined by using three methods: inhibitory activity toward the growth of test bacteria in vitro (7, 13, 14), inhibitory activity toward cell association, and invasion of pathogens using cultured human intestinal cells (6, 7, 12–14, 27), as well as protection of conventional or germfree mice against bacterial infection (7, 13, 14, 27). These showed how antimicrobial activities observed by in vitro methods could be confirmed in vivo as well.

In the present study, the Caco-2 cell line was used to study the adhesive properties of 47 potentially probiotic cultures in vitro. The cultures were also examined for antimicrobial properties toward pathogenic bacteria along with tolerance to low pH and bile salts. Among these cultures, five promising strains were examined by in vivo studies. The abilities of the selected strains to survive passage through the gastrointestinal tract and maintain colonization was tested in fecal samples using API 50CHL and internal transcribed spacer PCR (ITS-PCR) for primary selection of strains and restriction enzyme analysis (REA) combined with pulsed-field gel electrophoresis (PFGE) for confirmation of isolates recovered from fecal samples during and after administration. It was the main objective of this study to compare the in vitro evaluation of certain properties of various *Lactobacillus* spp. that are important for their survival in the gastrointestinal tract with their actual ability to survive in vivo.

MATERIALS AND METHODS

Bacterial strains and culture conditions. This study comprised 47 strains of *Lactobacillus* spp. (Table 1), of which 10 were collected from Ghanaian fermented maize, 11 had documented properties, 8 were human clinical isolates,

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TABLE 1. Origins of the lactobacilli tested in this study

Strain	Origin (reference)	Source
Isolates from Ghanaian fermented maize		
L. plantarum Lb1	Cassava, Ghana	M. Halm[a]
L. fermentum Lb6	Maize dough, Ghana	M. Halm
L. fermentum Lb11	Maize dough, Ghana	M. Halm
L. fermentum Lb12	Maize dough, Ghana	M. Halm
L. fermentum Lb18	Maize dough, Ghana	M. Halm
L. fermentum Lb20	Maize dough, Ghana	M. Halm
L. fermentum Lb41	Maize dough, Ghana	M. Halm
L. fermentum Lb43	Maize dough, Ghana	M. Halm
L. fermentum Lb48	Maize dough, Ghana	M. Halm
L. plantarum Lb50	Gari, Ghana	M. Halm
Strains with documented properties		
L. johnsonii (*acidophilus*) LA1	Human (6)	Nestlé[b]
L. crispatus BG2FO4	Human adult feces (11)	Klaenhammer[c]
L. acidophilus NCFM#2	Human (24)	Klaenhammer
L. paracasei subsp. *paracasei* DSM 20312	Human (44)	DSMZ[d]
L. rhamnosus 271	Human colon (28)	Molin[a]
L. plantarum 299	Human colon (28)	Molin
L. plantarum 299v	Sourdough (28)	Molin
L. reuteri R2LC	Rat colon (28)	Molin
L. reuteri DSM 12246	Pig feces (19)	El-Ziney[f]
L. rhamnosus LGG	Human adult feces (23)	Valio[g]
L. rhamnosus Lc705	Unknown (35)	Wiesbye[h]
Human clinical isolates		
L. plantarum 22319-21	Feces of healthy Ghanaian	MT[i]
L. acidophilus 18911-2	Feces of Danish child <1 yr old	MT
L. paracasei subsp. *paracasei* 19015-6	Feces of Danish child <1 yr old	MT
L. rhamnosus 19020-8	Feces of Danish child <1 yr old	MT
Lactobacillus sp. strain 19020-10	Feces of Danish child <1 yr old	MT
L. paracasei subsp. *paracasei* 19058-4	Feces of Danish child <1 yr old	MT
L. rhamnosus 19070-2	Feces of Danish child <1 yr old	MT
L. acidophilus 22571-8	Feces of healthy Ghanaian	MT
Dairy strains		
L. acidophilus CHCC 2169	Unknown	CHCC[j]
L. acidophilus CHCC 2168	Unknown	CHCC
L. crispatus CHCC 3692	Unknown	CHCC
L. crispatus CHCC 3577	Unknown	CHCC
L. rhamnosus CHCC 2100	Unknown	CHCC
L. rhamnosus CHCC 2099	Unknown	CHCC
L. delbrueckii subsp. *bulgaricus* CHCC 759	Unknown	CHCC
L. delbrueckii subsp. *bulgaricus* CHCC 2164	Unknown	CHCC
L. delbrueckii subsp. *lactis* CHCC 2329	Unknown	CHCC
L. helveticus CHCC 627	Unknown	CHCC
L. paracasei subsp. *paracasei* CHCC 2166	Unknown	CHCC
L. paracasei subsp. *paracasei* CHCC 3981	Unknown	CHCC
L. paracasei subsp. *paracasei* CHCC 3982	Unknown	CHCC
L. paracasei subsp. *paracasei* CHCC 3740	Unknown	CHCC
L. casei CHCC 3136	Unknown	CHCC
L. acidophilus CHCC 3264	Unknown	CHCC
L. casei subsp. *alactus* CHCC 3137	Unknown	CHCC
L. acidophilus Lb145	Unknown	Wiesbye

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and 18 were dairy strains. As indicator bacteria for the antimicrobial activity assay, both pathogenic and nonpathogenic normal habitants of the gastrointestinal tract were used (for details, see Tables 4 and 5). The nonpathogenic indicator bacteria were received from the Department of Clinical Microbiology, University Hospital of Copenhagen, Denmark, and the pathogenic indicator bacteria were kindly supplied by the Department of Veterinary Microbiology at the Royal Veterinary and Agricultural University, Frederiksberg, Denmark, except for the *Shigella flexneri* strain received from Statens Serum Institute, Copen-

hagen, Denmark. The lactic acid bacteria were grown in de Man, Rogosa, and Sharpe (MRS) broth (Merck), and the non-lactic acid bacteria were grown in brain heart infusion broth (Difco) at 37°C for 24 h. For long-term storage, the bacteria were kept at −40°C in 15% glycerol. All strains were subcultured twice prior to the experiments.

Cell culture. Enterocyte-like Caco-2 cells were kindly supplied by Derek Brown (Department of Veterinary Microbiology, Royal Veterinary and Agricultural University). Cells were routinely grown in Eagle's minimum essential medium (MEM) (MEM enriched with Glutamax and HEPES; Gibco Bethesda Research Laboratories [BRL]) supplemented with 10% heat-inactivated (30 min at 56°C) fetal bovine serum (Gibco BRL), 0.1 mM nonessential amino acids (Gibco BRL), and 0.5 ml of gentamicin (50 mg/ml) (Gibco BRL) and incubated at 37°C in a water-jacketed incubator with 5% carbon dioxide. Cells were used for adherence assay at postconfluence. Concentration of Caco-2 cells in the monolayer was determined by trypinizing the cells for 10 min at 37°C and counting them in a hemocytometer. Amounts of 3 ml containing 1.5×10^5 cells/ml were transferred to 35-mm-diameter dishes (Nunclon) and incubated until a complete monolayer was obtained. Change of medium was performed every 48 h.

Adhesion assay. Caco-2 cells in a monolayer were washed twice with phosphate-buffered saline, 3 ml of MEM was added to each dish, and the dishes were incubated for 30 min before inoculation of bacteria. Overnight cultures of bacteria were appropriately diluted (10×) with MEM to give a bacterial concentration of approximately 10^8 cells/ml, and 120 μl was used to inoculate the Caco-2 cells. After incubation for 1 h at 37°C, all of the dishes were washed four times with phosphate-buffered saline to release unbound bacteria. The cells were then fixed with 3 ml of methanol and incubated for 5 to 10 min at room temperature. After removal of the methanol, the cells were stained with 3 ml of Giemsa stain solution (1:20) (Merck, Darmstadt, Germany) and left to incubate for 30 min. The dishes were washed until no color was observed in the washing solution, dried in an incubator at 37°C overnight, and examined microscopically (magnification, ×100) under oil immersion. Each adhesion assay was performed in duplicate with cells from three successive passages (8 to 13 cell passages). The adherent lactobacilli in 20 random microscopic fields were counted for each test. Bacterial strains were scored as nonadhesive when fewer than 40 bacteria were present in 20 fields, adhesive with 41 to 100 bacteria in 20 fields, and strongly adhesive with more than 100 bacteria in 20 fields.

Antimicrobial activity assay. For detection of antimicrobial activity, an agar spot test was used. The test was a modification of that described by Schillinger and Lücke (42). Test cultures were spotted (2 to 3 μl) on the surface of MRS agar containing only 0.2% glucose and 1.2% agar and incubated anaerobically (GasPak system; BBL Microbiology Systems, Cockeysville, Md.) for 24 h at 30°C to develop the spots. The inhibitory effect of MRS was tested as a negative control on each plate. A 100-μl volume of an overnight culture of the indicator bacteria was mixed with 7 ml of soft agar (0.7%), using MRS agar for the lactic acid bacteria and brain heart infusion agar for the non-lactic acid bacteria, and poured over the plate. The plates were incubated either anaerobically (lactic acid bacteria) or aerobically (non-lactic acid bacteria) at 37°C. After 48 h of incubation, inhibition zones were read. A clear zone of more than 1 mm around a spot was scored as positive. Each test was performed twice.

pH and bile tolerance. The tests were performed in round-bottom microwell plates (Nunclon). A 200-μl volume each of MRS (pH 2.5), MRS containing 0.3% oxgall, or normal MRS, each inoculated with the test bacteria at a level of 10^6 cells/ml, was tested in each of four wells. As a control, broth without inoculation was used. Changes in optical density at 620 nm (OD_{620}) were measured (Multiscan MCC 340; Labsystem) following 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, and 24 h of incubation at 37°C. Survival under the different conditions was tested after 4 h of incubation at 37°C and plating of 100 μl onto MRS agar.

In vivo trials. Cultures were administered to a group of 12 healthy men between 18 and 37 years of age. The study was designed as a double-blind crossover study taking place over three periods (I, II, and III) of 35 days each (Table 2). For the first 18 days, the volunteers were randomized to consume twice a day a mixture of either (i) strains DSM 12246 and 19070-2, (ii) strains LGG, CHCC 3137, and CHCC 2329 (with each strain being present at a concentration of 10^{10} CFU per dose), or (iii) a placebo. The freeze-dried granulates were produced by Chr. Hansen A/S (Hørsholm, Denmark), except for the granulate containing strain LGG, which was produced by Valio (Helsinki, Finland). After the administration period, there was a washout period of 17 days (days 19 to 35). Thereafter, the person entered a second administration and washout period, followed by a third administration and washout period. Fecal samples were collected and investigated at days 0, 18, 23, and 29 of each period. The participants were told not to change their regular diets, but they abstained from fermented milk products. None of the participants received antibiotic treatment from 7 days before the study until the end. The study was approved by the Medical Ethical Committee of Copenhagen and Frederiksberg (KF 388/97) and The Danish Medicines Agency.

Microbial analysis of feces and reisolation of test strains. Fecal samples were kept at 5°C until 3 days before they were analyzed. Tenfold serial dilutions of the fecal samples were prepared with physiological saline, and 0.1 ml of each dilution was plated onto MRS agar (Merck). The plates were incubated at 37°C for 4 days in an anaerobic chamber. Representative colonies were selected on the basis of colony morphology, microscopy, Gram staining, and vancomycin resistance. The isolates were identified by API 50CHL (BioMerieux, Marcy l'Etoile, France) and ITS-PCR, with a final identification including restriction enzyme analysis and PFGE as described below.

TABLE 2. Design of in vivo trials[a]

Period and process	Strains given to volunteers:		
	1, 4, 7, 10	2, 5, 8, 11	3, 6, 9, 12
I			
Intervention[b]	C	A	B
Washout[c]			
II			
Intervention	A	B	C
Washout			
III			
Intervention	B	C	A
Washout			

[a] Fecal samples were taken at days 5, 18, 23, and 29 in each period. Strains were administered to 12 healthy male volunteers as follows: A, DSM 12246 and 19070-2; B, LGG, CHCC 3137, and CHCC 2329; C, placebo. For descriptions of strains, see Table 1.
[b] Days 1 to 18.
[c] Days 19 to 35.

ITS-PCR. The DNA was isolated from 1.5 ml of an overnight culture pretreated by heating at 65°C for 15 min in accordance with the manufacturer's instructions by using Dynabeads DNA DIRECT System I (Dynal, Oslo, Norway).

The PCR was performed in a reaction volume of 50 μl containing 1× *Taq* polymerase buffer (Pharmacia Biotech, Uppsala, Sweden), 1.5 U of *Taq* polymerase (Pharmacia Biotech), 0.5 μM each primer (16S-1500-Cy5 and 23S-32), 200 μM deoxynucleoside triphosphates, 2 mM $MgCl_2$, 1% (vol/vol) formamide, and 1 μl of the isolated DNA. The mixtures were subjected to 5 min at 94°C; 10 cycles of 30 s at 94°C, 30 s at 48°C, and 30 s at 72°C; 25 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and 5 min at 72°C, all in a TRIO Thermoblock (Biometra GmbH, Göttingen, Germany). For automatic analyses of ITS fragment length, the PCR products were separated by polyacrylamide gel electrophoresis on the A.L.F. express (Pharmacia Biotech). Electrophoresis was performed at 700 V and 55°C for 300 min. A size marker (sizer 50-500; Pharmacia Biotech) was included, and peak positions were analyzed in Fragment Manager (Pharmacia Biotech).

REA and analysis by PFGE. The PFGE protocol used was developed by Anette Wind (45) especially for lactobacilli. The OD_{600} of overnight cultures in MRS was measured, and 0.3 ml of each was centrifuged at 5,000 × *g* for 10 min and washed with 1 ml of SE buffer (75 mM NaCl, 25 mM EDTA pH 7.4). Cells were resuspended in SE buffer corresponding to a OD_{600} of 3.0, mixed with an equal volume of 2% low-melting-point agarose (Sigma), and imbedded in agarose plugs (Bio-Rad Laboratories, Hercules, Calif.). The agarose plugs were incubated in 0.5 ml of a solution containing 50 mM EDTA (pH 8.5), 0.05% *N*-lauroylsarcosine (Sigma), 2 mg of lysozyme (Sigma) per ml, and 3 U of mutanolysin (Sigma) per ml for 16 h at 37°C and then incubated overnight at 53°C in 0.5 ml of a solution containing 10 mM Tris, 0.5 M EDTA (pH 8.5), 1% sodium dodecyl sulfate, and 2 mg of proteinase K per ml. To remove the proteinase K, the agarose plugs were washed five times for 30 min each time in 1.5 ml of 50 mM EDTA (pH 8.5). Prior to restriction enzyme digestion, a part of the agarose insert (5 by 2.5 by 1 mm) was incubated in 0.25 ml of restriction enzyme buffer (NEBuffer 4 plus bovine serum albumin; New England Biolabs, Beverly, Mass.) for 1 h. The restriction enzyme digestion was performed in 0.1 ml of restriction enzyme buffer with 15 U of *Apa*I (New England Biolabs) and incubation for 16 h at 25°C. PFGE was performed with a CHEF-DRII (Bio-Rad Laboratories) on 1% agarose in 0.5 × TBE buffer (1 M Tris base, 0.83 M boric acid, 10 mM EDTA) at 14°C. The gel was run for 20 h at 200 V with a pulse time of 1 to 15 s.

RESULTS

Adhesion assay. Forty-seven *Lactobacillus* strains were examined for the ability to adhere to Caco-2 cells (Table 3). Considerable variation among the bacteria was observed. Strains 299, 299v, DSM 12246, LGG, 18911-2, 19020-10, 19070-2, CHCC 2329, CHCC 3137, and BG2FO4 were strongly adhesive, while the rest showed moderate-to-low adhesion.

Testing of antimicrobial properties. The antimicrobial properties of the *Lactobacillus* strains tested were very variable too

TABLE 3. Adhesion properties and pH and bile tolerances of 47 *Lactobacillus* strains

Lactobacillus strain	Adhesion[a]	Growth/survival[b]	
		pH 2.5	0.3% oxgall
Isolates from Ghanaian fermented maize			
Lb1	21 ± 14	−/+	**/+
Lb6	50 ± 41	−/+	**/+
Lb11	52 ± 23	−/+	**/+
Lb12	2 ± 2	−/+	**/+
Lb18	0 ± 0	−/+	***/+
Lb20	39 ± 28	−/+	***/+
Lb41	19 ± 12	−/−	***/+
Lb43	8 ± 3	−/+	**/+
Lb48	3 ± 4	−/+	*/+
Lb50	5 ± 5	−/+	*/+
Strains with documented properties			
LA1	17 ± 9	−/−	−/+
BG2FO4	272 ± 88	−/−	−/+
NCFM#2	26 ± 13	−/+	**/+
DSM 20312	2 ± 1	−/+	ND/ND
271	120 ± 19	−/+	**/+
299	268 ± 101	−/+	**/+
299v	355 ± 77	−/+	**/+
R2Lc	46 ± 39	−/+	ND/ND
DSM 12246	218 ± 116	−/+	**/+
LGG	630 ± 275	−/+	−/+
Lc 705	16 ± 15	−/+	**/+
Human clinical isolates			
22319-21	2 ± 1	−/+	−/+
18911-2	220[c]	−/+	−/+
19015-6	82 ± 26	−/+	−/+
19020-8	84 ± 26	−/+	−/+
19020-10	214 ± 87	−/−	−/+
19058-4	41 ± 11	−/−	−/+
19070-2	713 ± 188	−/+	−/+
22571-8	1 ± 1	−/−	−/−
Dairy strains			
CHCC 2169	5 ± 0	−/ND	−/ND
CHCC 2168	73 ± 7	−/+	−/+
CHCC 3692	17 ± 1	−/+	−/+
CHCC 3577	78 ± 3	−/−	−/+
CHCC 2100	72 ± 21	−/+	−/+
CHCC 2099	90 ± 16	−/+	−/+
CHCC 759	0 ± 0	−/ND	−/ND
CHCC 2164	16 ± 6	−/ND	**/ND
CHCC 2329	164 ± 14	−/−	−/+
CHCC 627	73 ± 7	−/−	−/+
CHCC 2166	22 ± 0	−/−	**/+
CHCC 3981	4 ± 4	−/−	−/+
CHCC 3982	1 ± 1	−/−	−/+
CHCC 3740	64 ± 5	−/−	−/+
CHCC 3136	65 ± 12	−/−	−/+
CHCC 3264	2 ± 2	−/−	−/+
CHCC 3137	551 ± 223	−/−	−/+
Lb145	20 ± 3	−/+	*/+

[a] Average number of adhering lactobacilli in 20 microscopic fields ± the standard deviation ($n = 3$).

[b] ***, no delay in growth; **, growth delay of <4 h; *, growth delay of >4 h; −, no growth or survival. ND, not determined.

[c] $n = 1$.

(Table 4). Many of the strains showed weak or no inhibition of the pathogenic strains. However, some strains, especially DSM 12246 and CHCC 2329, but also Lb1, Lb145, Lc705, 299, 299v, LGG, 22319-21, 19015-6, 19070-2, 22571-8, CHCC 3577, CHCC 2100, CHCC 2099, CHCC 2166, CHCC 3740, CHCC 3137, LA1, and BG2FO4, inhibited the pathogenic bacteria broadly. No inhibitory effect of MRS on any of the pathogenic strains tested was observed.

Different *Lactobacillus* spp., together with *Klebsiella oxytoca*, *Proteus mirabilis*, *E. coli*, *Citrobacter freundii*, *Enterobacter cloacae*, *Enterococcus faecalis*, and *Enterococcus faecium*, all being normal residents of the gastrointestinal tract, were tested as antimicrobial activity indicators toward selected strains. As can be seen from Table 5, strain DSM 12246 was without any inhibitory influence on the strains tested. LGG showed only minor antimicrobial inhibition toward *K. oxytoca* and *E. cloacae*. Strains CHCC 2329, CHCC 3137, 299, 299v, 271, and 19070-2 inhibited the normal residents of the intestinal flora more broadly. None of the eight lactobacilli tested as described above showed self-inhibition, nor did they inhibit each other (results not shown).

pH and bile resistance. Survival following 4 h of incubation at pH 2.5 was observed for 29 of the 44 strains tested, but none seemed to replicate (Table 3). The strains studied showed relatively high resistance to bile salts. Growth was delayed from 1 h to more than 4 h for 16 of the strains examined. The rest of the strains tested did not replicate, but all except 22571-8 survived for 4 h in 0.3% oxgall.

Recovery of lactobacilli from humans after intake. Cultures for the in vivo trial were selected primarily for their adhesive, as well as antimicrobial, properties, with LGG, 19070-2, and CHCC 3137 adhering strongly to Caco-2 cells and at the same time showing inhibition of the majority of the bacteria tested, whereas DSM 12246 and CHCC 2329 both expressed antimicrobial activities toward all of the bacteria tested along with good adhesion. Strains 299 and 299v showed promising results too, but they were not available for these trials. Table 6 shows that *Lactobacillus reuteri* DSM 12246 and *L. rhamnosus* 19070-2 and LGG were identified most frequently in the fecal samples from the volunteers. The bacteria were reisolated mainly during the period of administration (day 18), with 19070-2 being reisolated in 10 of 12 cases, DSM 12246 in 8 of 12 cases, and LGG in 7 of 12 cases by the REA-PFGE method used for final confirmation. The bacteria were generally reisolated at concentrations of 10^5 to 10^8 CFU/g of feces.

Reisolation was less frequent in the washout period, with DSM 12246 being identified in only one case and 19070-2 and LGG being identified in two cases each at day 23. No bacteria were reisolated from feces at day 29 (Table 6). Agreement among the three methods of identification was generally observed, although ITS-PCR detected some bacteria that were not confirmed by API 50CHL and PFGE (results not shown). ITS-PCR could not differentiate between DSM 12246 and CHCC 2329 (results not shown).

DISCUSSION

In vitro studies on bacteria have been used to evaluate various characters of potentially probiotic bacteria. Among these, tolerance of the low pH of the stomach and the bile content of the upper parts of the intestines and the ability to colonize the intestinal tract seem to be very important. Adherent strains are preferred, since their establishment in the intestines seems to be necessary for the probiotic effects to be exerted (34). In this study, adhesion to Caco-2 cells was found to be a discriminative parameter, showing pronounced variation among the strains, independent of the species. This variation among *Lactobacillus* spp. has been observed before (10, 43), including variation depending on the cell culture used (40). Earlier re-

TABLE 4. Antimicrobial activities of 47 lactobacilli toward pathogenic bacteria

Lactobacillus strain	Inhibition[a]						
	L. monocytogenes	*S. aureus*	*B. cereus*	*E. coli*	*S. typhimurium*	*S. flexneri*	*Y. enterocolitica*
Isolates from Ghanaian fermented maize							
Lb1	+	+	+	+	+	+	++
Lb6	−	−	+	−	−	−	+
Lb11	−	−	−	−	−	−	+
Lb12	−	−	−	−	−	−	+
Lb18	+	+	+	−	−	+	+
Lb20	−	−	−	−	−	−	+
Lb41	+	+	+	−	−	+	+
Lb43	+	−	−	−	−	−	+
Lb48	+	+	−	−	−	−	+
Lb50	−	−	+	+	−	−	+
Strains with documented properties							
LA1	+	+	+	+	+	+	++
BG2FO4	+	+	−	−	+	+	++
NCFM#2	+	−	−	−	−	+	++
DSM 20312	+	−	+	+	−	−	+
271	+	−	++	+	−	−	+
299	+	−	+	+	−	+	++
299v	+	−	+	+	−	−	+
R2Lc	+	−	+	−	−	−	+
DSM 12246	++	++	++	++	++	++	+
LGG	+	−	++	+	−	+	+
Lc705	+	+	+	+	−	+	++
Human clinical isolates							
22319-21	+	+	+	+	+	+	+
18911-2	+	−	+	−	−	+	++
19015-6	+	−	++	+	+	+	+
19020-8	+	−	++	+	−	−	+
19020-10	−	−	+	+	−	−	+
19058-4	+	−	+	+	−	−	+
19070-2	+	−	++	+	−	−	++
22571-8	+	+	+	+	+	+	++
Dairy strains							
CHCC 2169	+	−	++	+	−	−	+
CHCC 2168	+	−	+	+	−	−	+
CHCC 3692	+	−	+	−	−	+	+
CHCC 3577	+	−	++	+	+	+	−
CHCC 2100	+	−	++	+	−	+	++
CHCC 2099	+	−	++	+	−	+	++
CHCC 759	++	−	+	−	−	+	++
CHCC 2164	−	−	−	−	−	−	+
CHCC 2329	+	+	+	+	+	++	++
CHCC 627	+	−	+	+	−	−	+
CHCC 2166	+	−	+	+	−	+	+
CHCC 3981	+	−	++	+	−	−	++
CHCC 3982	+	−	+	+	−	−	+
CHCC 3740	+	−	++	+	+	−	++
CHCC 3136	+	−	+	−	−	+	+
CHCC 3264	+	−	+	+	−	+	++
CHCC 3137	+	−	++	+	+	+	+
Lb145	+	+	+	−	+	+	++

[a] +, Between 2 and 5 mm of inhibition; ++, 5 mm of inhibition and above; −, no inhibition. Abbreviations: *S. aureus*, *Staphylococcus aureus*; *B. cereus*, *Bacillus cereus*; *Y. enterocolitica*, *Yersinia enterocolitica*.

ports of tendencies of strains BG2FO4 (11, 24) and LGG (10, 18) to adhere to Caco-2 cells were confirmed in this study, whereas LA1, surprisingly, was found to be nonadhesive, in contrast to earlier studies (6), although the methods used are similar.

The ability to inhibit the growth of pathogenic bacteria varied broadly among the strains too, and together with adhesion to Caco-2 cells, this character was used to select potentially probiotic bacteria for the in vivo trial. *L. reuteri* DSM 12246 showed strong inhibition of all of the pathogenic bacteria tested. This strain is a known producer of reuterin (19), which could account for this antimicrobial activity. On the contrary, it seems that the normal intestinal flora tested was unaffected by DSM 12246 in vitro. If this characteristic is transferable to in

TABLE 5. Antimicrobial activities of eight selected lactobacilli toward normal bacterial residents of the gastrointestinal tract

Gastrointestinal bacterium	Antimicrobial activity[a]							
	19070-2	LGG	299v	299	DSM 12246	271	CHCC 3137	CHCC 2329
Lactobacillus sp.	+	–	–	+	–	+	–	+
Lactobacillus sp.	+	–	+	+	–	–	+	+
Klebsiella oxytoca	+	+	+	+	–	+	+	+
Proteus mirabilis	–	–	+	+	–	+	+	+
Escherichia coli	–	–	–	–	–	–	–	+
Citrobacter freundii	+	–	+	+	–	+	+	++
Enterobacter cloacae	+	+	+	+	–	+	+	–
Enterococcus faecalis	–	–	+	+	–	–	+	–
Enterococcus faecium	–	–	–	–	–	+	–	–

[a] +, between 2 and 5 mm of inhibition; ++, 5 mm of inhibition and above; –, no inhibition.

vivo conditions, it seems beneficial for the maintenance of the intestinal microflora.

In the present study, similar tolerances to 0.3% bile acid were observed among the strains tested. Therefore, this test was not used for selection of strains for further investigations. Gilliland and coworkers (22) used the same value to distinguish the bile tolerances of different strains. A more pronounced variability in resistance to bile salts has been shown by Chateau and colleagues (8), with all strains showing delayed growth (9).

The pH of 2.5 used in the present study seemed to be more damaging to the bacteria, with only 29 of 44 strains surviving 4 h of exposure and none of them growing. In another study (26), the pH tolerance of *L. acidophilus* La5 and *Bifidobacterium* strain Bb-12 was analyzed between pH 1 and pH 4, with the largest difference in survival being observed between pH 2 and pH 3.

Reisolation of the bacteria from feces in this study was based upon the use of ITS-PCR and API 50CHL for primary identification of potential reisolates and REA-PFGE for confirmation. Other studies have found the ITS-PCR method to be very effective for discrimination of different strains of *L. helveticus* (17), as well as strains of *Bifidobacterium* (33), but in this case, ITS-PCR could not differentiate properly among the strains. Phenotypic characterization by API 50CHL should be used mainly to select strains for further specific characterization too, as also stated by Johansson and coworkers (28). In the latter study, isolates of strain 299v were recovered in fecal samples from 8 of 13 volunteers 11 days after the administration had ended by plasmid analysis and REA for final identification, and

TABLE 6. Reisolation from feces of five different lactobacilli fed to 12 volunteers from day 1 to day 18, followed by a washout period lasting from day 19 to day 35

Day and *Lactobacillus* strain	Rate of reisolation (log no. of bacteria/g of feces) from volunteer no.:											
	1	2	3	4	5	6	7	8	9	10	11	12
0												
DSM 12246	–[a]	–	–	–	–	–	–	ND[b]	–	–	–	–
19070-2	–	–	–	–	–	–	–	ND	–	–	–	–
CHCC 2329	–	–	–	–	–	–	–	–	–	–	–	–
CHCC 3137	–	–	–	–	–	–	–	–	–	–	–	–
LGG	–	–	–	–	–	–	–	–	–	–	–	–
18												
DSM 12246	–	6.7	7.5	–	5.9	–	7.7	6.4	–	5.7	6.7	6.7
19070-2	8.2	7.7	7.4	5.0	5.8	–	4.7	6.7	–	7.7	7.5	5.8
CHCC 2329	7.6	–	–	–	–	–	–	–	5.6	–	6.7	–
CHCC 3137	–	–	–	7.9	6.2	–	–	–	–	5.7	–	–
LGG	–	5.0	–	7.6	7.7	6.0	–	6.0	5.0	–	7.7	–
23												
DSM 12246	6.7	–	–	–	–	–	–	–	–	ND	–	–
19070-2	–	–	–	5.0	–	–	–	7.7	–	ND	–	–
CHCC 2329	–	–	–	–	–	–	–	–	–	–	–	–
CHCC 3137	–	–	–	–	–	–	–	–	–	–	–	–
LGG	5.0	–	–	–	–	–	–	–	–	–	5.0	–
29												
DSM 12246	ND	–	–	–	–	–	–	–	–	–	–	–
19070-2	ND	–	–	–	–	–	–	–	–	–	–	–
CHCC 2329	–	–	–	–	–	–	–	–	–	–	–	–
CHCC 3137	–	–	–	–	–	–	–	–	–	–	–	–
LGG	–	–	–	–	–	–	–	–	–	–	–	–

[a] –, no reisolations.
[b] ND, not determined.

in another study, strain LGG was reisolated in fecal samples by using phenotypic identification in 6 of 18 volunteers up to 7 days after administration had ceased (23). In a recent study, strain LGG was reisolated at low levels from biopsies but only in a few cases in fecal samples from volunteers at 7 days after the treatment had been terminated (2).

Among the five strains tested in the in vivo trials, strains 19070-2, DSM 12246, and LGG were most frequently reisolated from fecal samples during the administration period. This indicates that these three strains survive better in the gastrointestinal tract than do the other two strains in the in vivo study (CHCC 3137 and CHCC 2329). Compared to the in vitro results, it seems that 19070-2, DSM 12246, and LGG survive passage through the intestinal tract mainly because of their good adhesion properties. CHCC 3137 and CHCC 2329 both showed good adhesion properties (especially CHCC 3137) but also a lower pH tolerance than the other three strains tested (Table 3); this probably explains their low survival in feces. The concentrations of the reisolated cultures were similar to those in other studies. LGG was detected at levels of 1.5×10^6 to 1.2×10^5 CFU/g of feces at days 5 and 7 of administration, respectively, following administration of an oral dose of 1.2×10^{10} CFU/day (41), whereas strains 299 and 299v accounted for up to 30% of the total content of lactobacilli in rectal biopsies (5.2 log CFU/g of mucosa) following 10 days of administration of oatmeal soup containing 5×10^8 CFU/daily dose (28). The results obtained in the present study demonstrate how strains 19070-2, DSM 12246, and LGG survive passage through the gastrointestinal tract and can be reisolated during the administration period. However, prolonged colonization by any of the tested bacteria does not seem to occur.

In conclusion, this study has shown how in vitro methods can be used for prediction of the survival potential of lactobacilli in the human gastrointestinal tract. Survival seems to be strongly linked to adhesion to Caco-2 cells and tolerance to pH 2.5. However, even strong adhesive properties and pronounced pH tolerance seems not to result in colonization and persistence of the lactobacilli for any length of time after administration of the cultures had been terminated.

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