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Screening of Bile Resistance and Bile Precipitation in Lactic Acid Bacteria and Bifidobacteria

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ABSTRACT

A modification of the ecometric method was developed for a rapid screening of bile resistance in lactic acid bacteria and bifidobacteria. Validation of the MEM bile assay (modified ecometric method) was performed comparing the bile resistance index (RI_{bile}) and the bile resistance ratio (R%). Most *Bifidobacterium* strains assayed were bile sensitive (83.3%), while only 62.1% of lactobacilli showed that behavior. Some bifidobacterial strains (55.6%) showed a crystalline precipitate when grown on solid medium supplemented with 0.5% ox bile. The crystalline structures produced by *B. pseudolongum* CIDCA 531 were isolated and analyzed by optical and scanning electron microscopy, thin-layer chromatography, melting point, and specific cholesterol reactions. Those studies confirmed the presence of cholesterol in these crystalline structures. On the other hand, none of the lactobacilli and streptococci studied had the ability to produce crystalline precipitates.

Lactic acid bacteria (LAB) and bifidobacteria are normal components of the intestinal microflora in humans and animals and are frequently associated with health-promoting effects. For this reason, there has been increasing interest in elaborating food products containing these bacteria as dietary adjuncts *(6, 8, 33)*.

One of the beneficial effects that may result from human consumption of LAB and bifidobacteria is prevention of gastrointestinal disorders. This effect could be partially ascribed to the inhibitory power of LAB against pathogenic bacteria *(7, 11)* and can be used as a criterion for strain selection *(23)*.

A high level of serum cholesterol is generally considered as a risk factor for coronary heart disease and atherosclerosis. Several studies indicated that consumption of cultured dairy products reduced serum cholesterol levels in humans, rabbits, and rats *(17, 18, 35, 37)*. In vitro experiments with *Lactobacillus acidophilus (9)* and *Bifidobacterium bifidum (34)* suggested that cholesterol assimilation by those microorganisms might be the reason for the low cholesterol levels. However, other workers *(22, 27)* claimed that cholesterol removal from liquid media could be due to its coprecipitation with deconjugated bile salts at pH lower than 5.5, and that neither lactobacilli nor bifidobacteria were capable of assimilating cholesterol. Thari et al. *(38)* reported that cholesterol removal by *Bifidobacterium* strains in liquid media in the presence of bile probably occurred by both coprecipitation with deconjugated bile salts and assimilation by growing cells. Statistical analysis of in vitro studies showed no significant correlation between cholesterol assimilation, deconjugation of bile salts, and bile resistance *(39)*. Some bifidobacteria and lactobacilli are able to deconjugate esters of bile acids in vitro and in vivo *(10, 15, 16, 22)*. In order to guarantee their probiotic effect after administration, microorganisms should survive passage through the small intestine. The importance of bile in the selection of intestinal flora and its toxicity has been fully demonstrated *(2, 5, 20)*. A tolerance for bile is an essential criterion in the selection of microorganisms for probiotic formulation.

Different methodologies have been proposed for the screening of bile-resistant strains, such as growth kinetics by measure of optical density in liquid media supplemented with bile *(12)*. This method is time consuming when a large number of strains are to be tested. Moreover, it can lead to erroneous interpretations because bile precipitates at the pH reached in LAB cultures *(22, 27)*. This prompted us to develop an easy semiquantitative technique named the MEM bile assay, a modification of the ecometric method *(30)* previously developed to assess the quality of selective media. This method is based on the inoculation of standardized cultures on solid media with a sequential streaking technique leading to ever-decreasing numbers of colonies. As no loop reloading is made, there is a substantial decrease in bacterial growth on consecutive streaks. When growth is observed, a numerical value is assessed for each streak.

The aim of the present study was to evaluate the ecometric method as a tool for a rapid screening of bile-resistant and bile-precipitating strains.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. The species and source of 34 LAB and 36 bifidobacteria used in this study are listed in Tables 1 and 2. The stock culture collection was maintained at −80°C in 0.3 M sucrose. Bacteria were propagated twice in tryptone–phytone–yeast extract broth (TPY) before experimental use *(36)*.

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TABLE 1. *Bile resistance and bile precipitation in* Bifidobacterium *spp. strains evaluated by the MEM bile assay*

Strains		Source[a]	RI_{bile}[b]	Crystals[c]
B. pseudolongum	531	C	15	3
B. breve	15700	A	15	3
B. animalis	25527	A	15	1
B. breve	MB-19	B	12.5	2
B. infantis	15697	A	8	1
B. bifidum	539	D	8	2
B. longum	MB-56	B	6	2
B. adolescentis	5317	D	5	2
B. bifidum	5321	D	4	0
B. breve	5314	D	3.5	0
B. breve	5315	D	3.5	1
B. breve	5312	D	3	1
B. bifidum	5318	D	3	0
B. bifidum	533	D	3	2
B. bifidum	537	D	2	2
B. bifidum	5311	D	2	1
B. breve	532	D	2	2
B. longum	5316	D	2	2
B. dentium	29534	A	1	0
B. longum	5325	D	0.5	1
B. longum	5323	D	0.5	0
B. longum	5324	D	0.5	0
B. breve	5326	D	0.5	2
B. catenulatum	27539	A	0.5	0
B. infantis	MB-2	B	0.5	1
B. bifidum	5310	D	0.5	1
B. bifidum	5313	D	0.5	1
B. bifidum	536	D	0	0
B. longum	5319	D	0	0
B. longum	5320	D	0	0
B. longum	5322	D	0	0
B. breve	5327	D	0	0
B. infantis	538	D	0	0
B. infantis	MB-9	B	0	0
B. angulatum	27535	A	0	0
B. pseudocatenulatum	27919	A	0	0

[a] Isolated strains were identified by morphology, Gram staining, and biochemical characteristics according to *Bergey's Manual (36)* and whole protein pattern by sodium dodecyl sulfate polyacrylamide gel electrophoresis *(14)*. A, ATCC reference strain; B, obtained from Morinaga Milk Industry (Japan); C, isolated from a dairy product (CIDCA collection); D, isolated from healthy human newborn feces (CIDCA collection).

[b] RI_{bile}, bile resistance index; each value represents the average from three trials of the MEM bile assay.

[c] Presence of crystalline structures in three trials of the MEM bile assay.

TABLE 2. *Bile resistance and bile precipitation in* Lactobacillus *spp. and* Streptococcus salivarius *subsp.* thermophilus *strains evaluated by the MEM bile assay*

Strains		Source[a]	RI_{bile}[b]	Crystals[c]
L. delbrueckii subsp. *lactis*	133	E	15	0
L. delbrueckii subsp. *lactis*	135	E	15	0
L. plantarum	335	H	15	0
L. plantarum	337	H	15	0
L. gasseri	378	I	15	0
L. salivarius	433	F	15	0
L. delbrueckii subsp. *lactis*	1316	E	11.7	0
L. casei subsp. *rhamnosus*	LGG	C	10.8	0
L. delbrueckii subsp. *lactis*	1317	E	10	0
L. gasseri	374	I	8	0
L. acidophilus	139	E	8	0
L. delbrueckii subsp. *lactis*	137	E	5	0
L. delbrueckii subsp. *lactis*	335	C	4	0
L. delbrueckii subsp. *lactis*	1313	C	3.5	0
L. delbrueckii subsp. *lactis*	325	E	0	0
L. delbrueckii subsp. *lactis*	136	E	0	0
L. delbrueckii subsp. *lactis*	1313	E	0	0
L. delbrueckii subsp. *lactis*	1314	E	0	0
L. delbrueckii subsp. *lactis*	1315	E	0	0
L. delbrueckii subsp. *lactis*	222	E	0	0
L. delbrueckii subsp. *lactis*	132	E	0	0
L. delbrueckii subsp. *lactis*	134	E	0	0
L. delbrueckii subsp. *lactis*	MF 1	G	0	0
L. delbrueckii subsp. *lactis*	MF 2	G	0	0
L. delbrueckii subsp. *bulgaricus*	11842	A	0	0
L. delbrueckii subsp. *bulgaricus*	331	C	0	0
L. delbrueckii subsp. *bulgaricus*	332	C	0	0
L. delbrueckii subsp. *bulgaricus*	333	C	0	0
L. delbrueckii subsp. *bulgaricus*	334	C	0	0
S. salivarius subsp. *thermophilus*	19528	A	0	0
S. salivarius subsp. *thermophilus*	328	C	0	0
S. salivarius subsp. *thermophilus*	221	E	0	0
S. salivarius subsp. *thermophilus*	3212	C	0	0
S. salivarius subsp. *thermophilus*	3214	C	0	0

[a] Isolated strains were identified by morphology, Gram staining, and biochemical characteristics *(4)*. A, ATCC reference strain; C, isolated from dairy product (CIDCA collection); E, raw milk (CIDCA collection); F, fermented meat product—salami—(CIDCA collection); G, healthy human adult feces (CIDCA collection); H, provided by Dr. M. Daeschel (Dept. of Food Science and Technology, Oregon State University, Corvallis); I, provided by Dr. R. Raya (CERELA, Centro de Referencia para Lactobacilos, Tucumán, Argentina).

[b] RI_{bile}, bile resistance index; each value represents the average from three trials of the MEM bile assay.

[c] Presence of crystalline structures in three trials of the MEM bile assay.

Two percent inocula was used for lactobacilli and streptococci and 4% for bifidobacteria. Incubations were performed at 37°C for 20 to 24 h in anaerobic conditions. TPY broth contained (in g/liter): tryptone, 10.0; soy peptone, 5.0; glucose, 5.0; yeast extract 2.5; tween 80, 1.0; cysteine hydrochloride, 0.5; K_2HPO_4, 2.0; $MgCl_2 \cdot 6H_2O$, 0.5; $ZnSO_4 \cdot 7H_2O$, 0.25; $CaCl_2$, 0.15; pH 7.0. TPY agar containing ox bile as a selective agent (TPY–bile) was prepared by supplementing 9.0 ml of molten sterile media at 45 to 50°C with 1.0 ml of concentrated (10×) bile solution. Unless stated otherwise, final concentration of ox bile was 0.5%. Dehydrated ox bile (Merck & Co., Rahway, N.J.) containing cholesterol was dissolved in distilled water and sterilized by filtration with a 0.45-μm pore-size filter (Millipore, Molsheim, France). TPY–bile salts without cholesterol (0.5% bile salts) was prepared with rehydrated bile salts containing 50% cholic and 50% deoxycholic acids as sodium salts (Difco Laboratories, Detroit, Mich.). Solid media were prepared by adding 15 g of agar per liter. Sugars and salts were from Mallinckrodt Inc. (St. Louis, Mo.), and the other media components from Difco. Media were sterilized by auto-

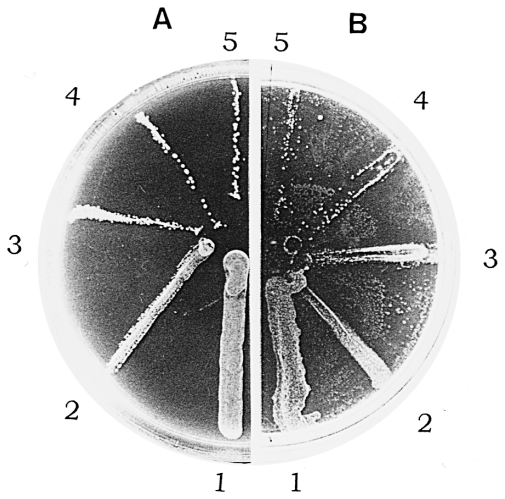

FIGURE 1. *Growth of* L. casei *subsp.* rhamnosus *(*Lactobacillus *GG) on TPY (A) and TPY–bile containing 0.5% ox bile (B). Five consecutive streaks are indicated by numbers 1 to 5.*

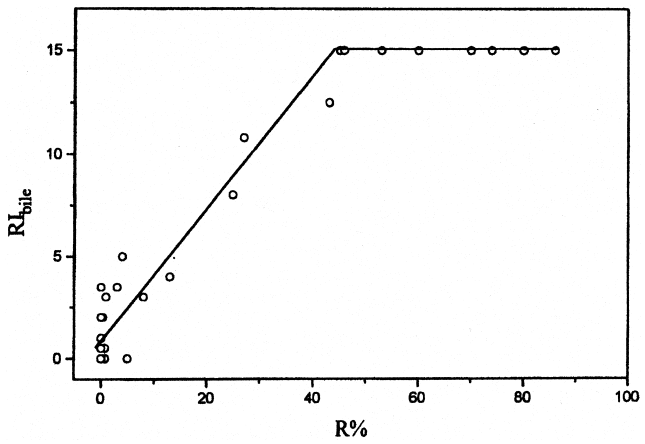

FIGURE 2. *Correlation of RI_{bile} and R% of 27 strains of LAB and bifidobacteria. Each point corresponds to the mean value of three trials of the MEM bile assay and colony counts performed with each strain.*

claving at 121°C for 15 min. All media were stored at 4°C for a maximum of 7 days.

Measurement of bile resistance. Cultures in stationary phase (10^8 to 10^9 CFU/ml) were obtained after 20 to 24 h of incubation (OD_{600nm} = 1.1 to 1.2). Bile resistance was determined by means of two different methods: (i) Viable counts were determined on TPY agar in the presence (TPY–bile) and absence (TPY) of ox bile by plating appropriate dilutions made in 0.1% tryptone. Plates were incubated for 72 h at 37°C in anaerobic conditions. Experiments were performed in duplicates and only plates with 30 to 300 colonies were considered. The ratio of bile-resistant bacteria was calculated as follows:

$$R\% = [(CFU/ml)_{TPY-bile} \times 100]/(CFU/ml)_{TPY}$$

(ii) The MEM bile assay was performed by making five consecutive streaks on TPY agar and TPY–bile agar using a calibrated 5-μl platinum wire loop (Laboratorios Britania S.A., Los Patos, Buenos Aires, Argentina) without reloading or flame sterilization. Incubation was done as indicated previously, and growth was recorded for each streak. To achieve reproducible results the details of inoculation procedure recommended by Mossel et al. *(30)* have been considered. The degree of resistance was quantified by assigning a numeric value to the positive growth in each streak on TPY–bile. Only experiments with confluent growth in five streaks on TPY (control dishes) were considered. When confluent growth on TPY–bile was observed, the number assigned from the first to the last streak were 1, 2, 3, 4, and 5, respectively. However, when growth on TPY–bile was poor in comparison to TPY, the numbers assigned to each streak were a fraction of the corresponding number. Absence of growth on TPY–bile was recorded as zero. The bile resistance index, RI_{bile}, was calculated as the sum of the values assigned to the five streaks (maximum value for the first to the fifth streak were 1, 2, 3, 4, and 5, respectively). RI_{bile} ranged from 0 to 15. Correlation of R% and RI_{bile} was made by using least-squares analysis.

Physicochemical analysis of crystalline precipitates. Bacterial confluent growth with crystalline precipitates was obtained with some strains after incubation on solid TPY–bile for 72 h at 37°C in anaerobic conditions. Crystals were harvested, washed twice with deionized water, and centrifuged at 14,000 × *g* for 2 min (washed pellets). Control experiments were performed on TPY and TPY–bile salts without cholesterol.

Thin-layer chromatography (TLC). Pellets containing bacteria and crystals were extracted by vortexing during 1 min with 1 ml of chloroform. In those conditions crystals were partially solubilized in the solvent, crystals associated with bacteria remained in the upper phase. Forty microliters of concentrated (10×) chloroformic extracts of crystals as well as standard cholesterol (Merck) dissolved in chloroform (4×10^{-5} g) were spotted on 0.25-mm silica gel G layer plates T-6770 (Sigma Chemical Company, St. Louis, Mo.), previously activated at 100°C for 30 min. Chromatograms were run using an acetone/chloroform (9/1) solvent system at 30°C to a height of 8.0 cm in a glass tank lined with filter paper saturated with the solvent. After drying, cholesterol spots were developed by iodine vapor and then sprayed with a concentrated sulfuric acid spray reagent. Cholesterol spots stained red-brown after heating the plates at 110°C for 5 min. The Rfs of these spots and those of standard cholesterol were measured. All chemicals employed were from Sigma Chemical Co.

Microscopic examination. Electron microscopy examination was made by mounting washed pellets on slides, flame-fixing, and coating with gold-24 for 10 min by vacuum evaporation (Fine Coat, Ion Sputter JFC-1100, JEOL Ltd., Tokyo, Japan). A JSM-T100 electron microscope JEOL (JEOL Ltd.) was employed operating at 15 to 20 kV. Optical microscopy examination was made in a Leitz Ortholux II microscope (Ernst Leitz, Wetzlar, Germany). Slides with mounted pellets were also observed under polarized light using an L32/40 objective.

Melting point determination. Washed pellets were dried (24 h at 42°C), mounted on slides, and melting points were determined in Bock-Monoscop equipment (Karl Kolb, Dreieich, Germany). Temperature was gradually increased from 40 to 330°C at a rate of 10°C/min.

Salkowski reaction for cholesterol characterization. Washed pellets and standard cholesterol crystals were placed in a glass test tube containing 2 ml of chloroform, shaken vigorously by vortexing, and 2 ml of concentrated sulfuric acid was carefully added and mixed. A purple-red color in the chloroform phase is indicative of cholesterol *(40)*.

TABLE 3. *Bile resistance for probiotic bacteria using the MEM bile assay at different bile concentrations*

Genera	Strains tested	Percentage of bile-resistant strains at bile concentration		
		0.1	0.5	1.0
Lactobacillus	36	100	38.5[a]	18.2[a]
Bifidobacterium	29	100	15.1	3.8
Streptococcus	5	100	0	0

[a] No bile-resistent strains were found in five *Lactobacillus delbrueckii* subsp. *bulgaricus* tested.

Liebermann–Burchard reaction for cholesterol characterization. This assay is based on the strong color developed when cholesterol is combined with strong acids *(40)*. Extraction and quantification of cholesterol was performed according to Abell et al. *(1)*. Interfering substances like bile salts were eliminated by extraction with hexane where cholesterol is highly soluble and bile salts were not. Isolated crystals (300 mg) or standard cholesterol crystals were placed in a glass test tube containing 5 ml of ethanol. After adding 10 ml of hexane, the mixture was shaken vigorously by vortexing and centrifuged at 1,000 × *g* for 2 min. The upper hexane phase was concentrated by evaporation at 60 to 70°C under nitrogen. Cholesterol remained in the hexane phase and bile salts and bacteria in the ethanol phase. Concentrated cholesterol was mixed with a solution of acetic anhydride/concentrated sulfuric acid (30/1). Quantification of cholesterol in standard solutions (0.008 to 0.032 mg/ml of standard cholesterol and 3.5 mg/ml of bile salts) and cholesterol extracted from crystals by hexane was performed by measuring the OD_{620nm}. According to the calibration curve, an OD_{620nm} of 0.1093 ± 0.0019 is equivalent to 10 μg of cholesterol. Dry weight of washed crystals was determined after drying at 100°C until constant weight was achieved.

RESULTS

The MEM bile assay. Growth on the fourth to the fifth streak in TPY with ox bile was observed in *Lactobacillus* GG (Fig. 1). This strain has already been described as moderately bile resistant *(13)* and was considered in this paper as reference for the selection of other bile-resistant strains. A low degree of growth for bile-sensitive strains was found on TPY–bile (data not shown).

Validation of the MEM bile assay for classification of strains according to their bile resistance was performed by comparing RI_{bile} and R% obtained by plate count in TPY with or without 0.5% ox bile. Figure 2 shows the results obtained with 50 strains of different genera and species of LAB and bifidobacteria. A linear correlation between R% and RI_{bile} was obtained for R% values ranging from 0 to 45% ($R = 0.93$, $n = 18$) in three trials of plate count and MEM bile. *Lactobacillus* GG showed an R% of 27.0 ± 8.0 and an RI_{bile} of 10.8 ± 1.8, respectively. Strains with $RI_{bile} \geq 8.0$ were considered as bile resistant. This value of RI_{bile} corresponded to R% ≥ 25 at 0.5% ox bile. The maximum RI_{bile} for a resistant strain by the MEM bile assay was 15, so the curve showed the same RI_{bile} for strains with R% ≥ 45. Differentiation among these resistant strains could be obtained by performing a higher number of streaks. However, a clear distinction between sensitive and resistant strains was obtained with five streaks in the screening of bile-resistant strains ($RI_{bile} \geq 8.0$).

The MEM bile assay was performed with 70 strains on TPY supplemented with three different concentrations of ox bile: 0.1, 0.5, and 1.0% (Table 3). Bacterial growth was observed to be lower on TPY–bile than on TPY without bile even for the resistant strains. The degree of growth inhibition increased with the concentration of bile. Table 3 shows that all strains were resistant to 0.1% ox bile, including species already known as bile-sensitive like *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus (28)*. In contrast, only 2.2% of all strains studied was able to grow at 1.0% ox bile. Because 0.5% of ox bile allowed the growth of *Lactobacillus* GG and not the growth of nonintestinal LAB, this concentration was chosen for the screening of bile-resistant strains by MEM bile.

Tables 1 and 2 show that 6 out of 36 (16.7%) and 11 out of 29 (37.9%) strains of bifidobacteria and lactobacilli, respectively, were resistant to 0.5% ox bile. None of the five strains of streptococci studied could grow on 0.5% ox bile. Twenty-three out of 24 newborn human fecal isolates were bile sensitive, with the exception of *B. bifidum* CIDCA 539. Among the ATCC strains studied, *B. breve* 15700, *B. infantis* 15697, and *B. animalis* 25527 were resistant. Strain *B. pseudolongum* CIDCA 531, isolated from fermented milk, was highly resistant to ox bile.

Bile precipitate. Some of the strains screened by MEM bile showed crystalline precipitates of 0.5 to 1.0 mm on the growth surface of TPY–bile agar (Fig. 3A). These precipitates were absent on noninoculated TPY–bile agar, on inoculated TPY, and on inoculated TPY–bile salts media without cholesterol (data not shown). The crystalline structures produced by *B. pseudolongum* CIDCA 531 were isolated and analyzed by optical and scanning electron microscopy (Fig. 3B and 3C, respectively). These figures show bacteria as well as crystals of different sizes and shapes, such as tubules, filamentous, and the classical plate-like cholesterol monohydrate crystals (Fig. 3B). Using scanning electron microscopy (Fig. 3C), multilaminar structures typical of cholesterol stones and associated bacteria were observed. Table 4 shows that chloroform extracts of standard anhydrous cholesterol gave a unique spot on TLC of Rf = 0.79. Chloroform extraction of crystals produced by *B. pseudolongum* CIDCA 531 grown in TPY–bile also gave one spot with a slightly different Rf (Rf = 0.71). A similar spot with Rf = 0.71 was observed in chloroform

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FIGURE 3. *Crystalline precipitates containing cholesterol produced by strain* Bifidobacterium pseudolongum *CIDCA 531 grown on TPY–bile agar. Observation at different magnification: optical microscopy (A: 50×, bar = 400 μm; B: 320×, bar = 25 μm) and electron microscopy (C: 5,000×, bar = 4 μm).*

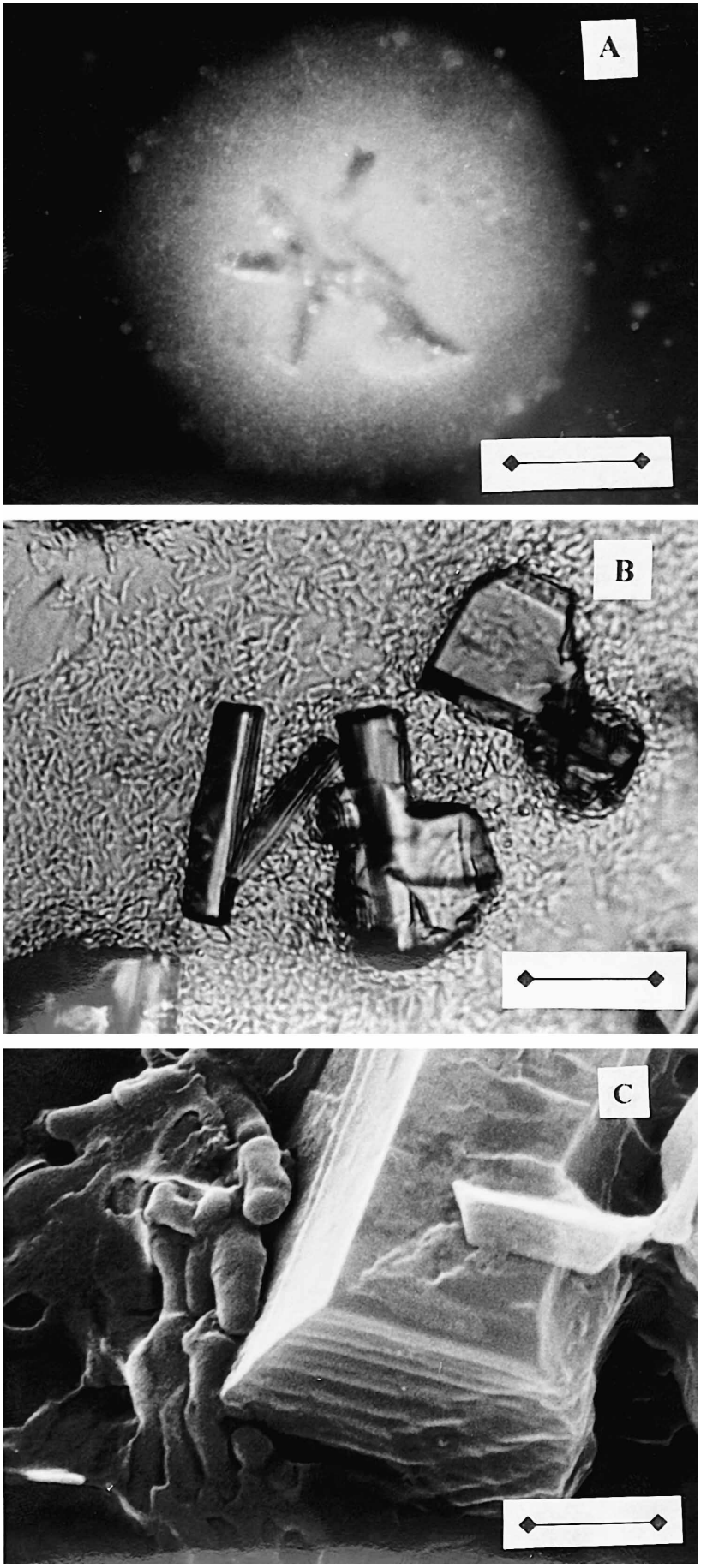

TABLE 4. *Physicochemical characterization of crystaline structures produced by* Bifidobacterium pseudolongum *strain CIDCA 531 on TPY–bile*[a]

Assay	Cholesterol[b]	Crystals
Solubility in chloroform	t	p
TLC (Rf)[c]	0.79	0.71
Melting point (°C)	148	147–150
Microscopic observation	pl[d]	pl, tu, fi[d]
Birrefringence	+	t
Liebermann–Burchard reaction	+	+
Salkowski reaction	+	+

[a] Each assay was performed in triplicate.
[b] Standard anhydrous cholesterol (Merck); t/p, total or partial solubility in chloroform.
[c] TLC (Rf), thin-layer chromatography, acetone/chloroform 1/1 (vol/vol) solvent system.
[d] pl, plate-like; tu, tubules; fi, filamentous.

extracts of ox bile containing cholesterol (data not shown). However, a chloroform extract of strain CIDCA 531 grown without bile did not show this spot. Melting point analysis of washed crystals showed a similar value to that obtained with anhydrous cholesterol. In addition, the crystalline structures showed birefringence and the cholesterol-positive reactions of Salkowski and Liebermann–Burchard. Crystalline structures could not be entirely dissolved in chloroform in contrast to anhydrous cholesterol. The Liebermann–Burchard reaction performed on bile salts without cholesterol gave a negative reaction, whereas ox bile gave a slightly positive reaction. The reaction obtained from crystals produced by strain CIDCA 531 was positive (OD_{620nm} = 0.0600 ± 0.0015). Considering the weight of extracted crystals, the amount of cholesterol is 0.10 μg/mg of crystal. This means that other substances are the main components of these crystals.

Twenty out of 36 *Bifidobacterium* strains (55.6%) were able to produce crystalline structures (Table 1). However, none of the strains of lactobacilli and streptococci showed crystals on solid TPY–bile (Table 2). The ability of bifidobacteria to produce crystals was a strain-related trait and was observed both in moderately sensitive strains and bile-resistant strains. Strains that did not show any growth (RI_{bile} = 0) were not able to produce crystalline structures. *B. pseudolongum* CIDCA 531 and *B. breve* ATCC 15700 showed the highest ability to produce crystals because the same results were obtained in the three trials of MEM bile.

DISCUSSION

The MEM bile assay is a modification of the ecometric method that can be used for the screening and selection of bile-resistant bacteria. A defined RI_{bile} parameter was used to determine semiquantitatively the bile resistance or bile sensitivity of LAB and bifidobacteria on TPY agar supplemented with 0.5% ox bile.

An ox bile concentration of 0.5% was found to be optimum for the screening of bile-resistant strains. That concentration was equivalent to 5.0 to 6.0% of whole physiological bile and was similar to that used by other workers in conditions that mimicked the small intestine environment *(28)*.

Validation of the MEM bile assay was supported by the good correlation between RI_{bile} and the R% obtained by means of viable counts on TPY with or without ox bile. Moreover, good reproducibility was obtained in assays performed in triplicate with the 70 strains, because for highly resistant strains with RI_{bile} = 15 and sensitive ones with $RI_{bile} \leq 0.5$ (66% of the strains in our study), the standard deviation (SD) was zero. In addition, the score chosen for quantifying the RI_{bile} allowed a clear distinction between resistant and sensitive strains. The use of *Lactobacillus* GG and the ATCC strains would allow interlaboratory comparisons to be made.

As other workers *(3, 12, 15, 20, 39)* have noted, we also observed that bile resistance is a strain-related trait. It is important to note that among the 29 strains of lactobacilli tested, strains isolated from healthy human adult feces (CIDCA MF1 and CIDCA MF2 belonging to *L. delbrueckii* subsp. *lactis*) were highly bile sensitive. Therefore, LAB isolated from human feces could be either bile resistant or bile sensitive.

We found a higher percentage of bile-resistant strains among *Lactobacillus* in comparison to *Bifidobacterium.* The difference between both genera can be explained because the lactobacilli are inhabitants of both the upper and lower intestinal tract (with high and low bile concentrations, respectively), while bifidobacteria are predominantly colonic inhabitants *(25)*.

In *Bifidobacterium,* among the 36 strains tested, 3 out of the 6 bile-resistant ones were ATCC strains: *B. infantis* 15697, *B. breve* 15700, and *B. animalis* 25527. Previous results have demonstrated that these strains were able to grow in liquid media supplemented with bile salts *(16, 20)*.

The high ratio of bile-sensitive strains among bifidobacteria isolated from human feces can be explained considering that they were isolated from newborns, in which intestinal colonization would have occurred before biliar secretion.

Both bifidobacteria and lactobacilli showed the ability to remove cholesterol from liquid media containing ox bile *(3, 9, 15, 19, 22, 34, 38, 39)*. A medium containing 0.5% ox bile had 68 μmol of cholesterol per liter *(22)*. Moreover, it is known that cholesterol is insoluble and precipitates in aqueous systems, forming ordered liquid crystals called cholesterolemic mesophases *(31)*.

We observed the presence of cholesterol-containing crystals in *B. pseudolongum* CIDCA 531 grown on TPY–bile (Table 3 and Fig. 3A). The absence of crystals on TPY–bile media incubated without bifidobacteria and in TPY cultures of bifidobacteria demonstrates that not only bile but also bacterial growth is necessary for crystal formation. By scanning electron microscopy, the standard technique for microscopic visualization of cholesterol stones, the typical multilaminar structure (Fig. 3C) already described for such stones *(26)* was observed. This result confirmed the presence of cholesterol in the crystals. Moreover, we observed that no precipitation occurred when such strains were incubated with bile salts without cholesterol,

indicating that the presence of cholesterol in the medium is necessary for these crystals to form.

Probably other bile compounds in ox bile or bacterial metabolites are also present in crystals, according to others reports *(22, 27, 38)*. This hypothesis is supported by the characteristics of the crystalline structures such as their incomplete solubility in chloroform, their low cholesterol content, Rf spot shift in TLC, slightly higher melting point, and presence of different crystalline shapes. Preliminary results showed the presence of bile acids in these crystalline structures (data not shown).

The ability to produce crystals is a strain-related characteristic and is not restricted to bile-resistant strains. Bile-resistant and moderately bile-sensitive strains were able to form crystals (Table 1). We showed that *Bifidobacterium* strains ATCC 15700 (*B. breve*), 15697 (*B. infantis*), and 25527 (*B. animalis*) were able to form crystalline structures. Moreover, strain ATCC 15700 produced crystals in all trials (Table 1). Similar results were obtained by Thari et al. *(38)* with these three strains.

A high percentage of bifidobacteria was able to produce crystalline structures probably because of the extracellular activity of bile hydrolase (BHS). This extracellular enzymatic activity splits conjugated bile salts—the main cholesterol solubilization vehicle in bile—and then cholesterol precipitation occurs. In contrast, none of the lactobacilli and streptococci strains tested in this study showed this property according to their intracellular BHS activity *(33)*.

The production of cholesterol-containing crystals of different shapes—filaments, tubules, and the classical plate-like cholesterol monohydrate crystals—were also observed by other workers during recrystallization of cholesterol from bile *(21, 24, 29, 32)*.

We believe that both the external composition and the bacterial arrangement play an important role in crystal formation as was observed for gallstone production. At this point, we have noticed an association between bacteria and crystals (see Fig. 3B and 3C). Some authors have assumed an interaction between cholesterol and the bacterial surface similar to that observed between bacteria and mutagenic substances *(19)*. This mechanism of cholesterol coprecipitation and association to the bacterial surface could explain the hypocholesterolemic effect of some probiotic bacteria.

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