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Expression of *Clostridium thermocellum* Endoglucanase Gene in *Lactobacillus gasseri* and *Lactobacillus johnsonii* and Characterization of the Genetically Modified Probiotic *Lactobacilli*

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Abstract. Endoglucanase A from *Clostridium thermocellum* resistant to pancreatic proteinase was selected out of a range of microbial cellulases expressed in *lactobacilli*. Two *Lactobacillus–E. coli* expression vectors, harboring the endoglucanase gene from *C. thermocellum* under the control of its own promoter (pSD1) and the *Lactococcus lactis* lac A promoter (pSD2), were constructed separately. Intestinal *Lactobacillus* strains, *L. gasseri* and *L. johnsonii*, were electrotransformed with pSD1 and pSD2, and the stability of each plasmid was evaluated. The endoglucanase activities of 0.722 and 0.759 U/ml were respectively found in culture medium of *L. gasseri* and *L. johnsonii* containing pSD1, and of 0.407 U/ml in medium of *L. gasseri* harboring pSD2. When the probiotic characteristics such as acid-tolerance, bile-salt tolerance, and antibiotic susceptibility were investigated, *L. gasseri* and *L. johnsonii* were resistant to low pHs of 2 and 3. Also, *L. johnsonii* was bile-salt resistant in the presence of 0.5% oxgall and porcine bile extract. *L. johnsonii* and *L. gasseri* showed a rather homogeneous resistant pattern against tested antibiotics. Both strains were resistant to amikacin, bacitracin, gentamicin, streptomycin, kanamycin, and colistin.

Lactobacillus species are normal inhabitants of the mammalian gastrointestinal tract and are main constituents of probiotics that are fed to newborn livestock with the aim of enhancing the immune system, increasing body weight gain, and improving feed conversion efficiency [1]. Recently, it was suggested that more efficacious probiotics can be developed through the genetic modification of gastrointestinal strains of *lactobacilli* [25]. The modified strains would colonize the digestive tract when the animal hosts consumed and synthesized a novel product [16]. But so far, the development of genetically modified *lactobacilli* has proved difficult, largely because suitable plasmid expression cassettes with strong promoters and signal sequences of *Lactobacillus* origin have not been established.

In the particular field of mono-gastric animal nutrition and health, the major plant cell wall components of cereals, primarily β-glucans and arabinoxylans, form gel-like structures in the small intestines, trapping nutrients, and therefore enzymatic hydrolysis and absorption are hindered [15]. The viscous polysaccharides can also cause severe gastrointestinal disorders [15]. To improve non-ruminant animal performance, barley-based diets are often supplemented with endoglucanases and endoxylanases, particularly in poultry. However, enzyme supplementation substantially increases the cost of feed and is used for only a short-term solution in enhancing digestion of cereals. An alternative and less expensive strategy might be designed to develop *lactobacilli* with the capacity to digest plant structural carbohydrates by the introduction of heterologous genes encoding polysaccharide-degrading enzymes.

The expression of cellulases and xylanases genes in *lactobacilli* is mainly described in silage starter bacteria, *L. plantarum* and little is known about other intestinal *lactobacillus* strains [2, 24].

Clostridium thermocellum, a Gram-positive thermophilic anaerobe, secretes a highly potent and thermo-

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stable cellulase complex [10]. The *cel* A gene codes for a previously purified endoglucanase(1,4-β-D-glucan glucanohydrolase EC 3.2.1.4.), endoglucanase A, which has an MW of 56,000 and is one of the major endoglucanases secreted by *C. thermocellum* [23].

In this work, we expressed the *C. thermocellum* endoglucanase gene in *L. gasseri* and *L. johnsonii* and also examined the probiotic characteristics such as acid tolerance, bile-salt tolerance, and antibiotic susceptibility for using these genetically modified *lactobacillus* strains.

Materials and Methods

Bacterial strains, plasmids, and media. *Escherichia. coli* MC 1061 [5] was used as a host for the construction of recombinant expression vectors. *L. gasseri* ATCC 33323 [30] and *L. johnsonii* NCK 88 [30] were used as hosts for studying endoglucanase expression. Plasmids pCT104 [3], pNZ123 [11], and pNZ3004 [29] were used for the construction of recombinant expression vectors. *Lactobacilli* were cultivated in MRS media (Difco) at 37°C. *Lactobacillus* transformants were selected on MRS plates with 0.5% CMC (carboxymethyl cellulose) solidified by 1.5% agar. Antibiotics were amended with 10 μg/ml chloramphenicol (Cm) and 50 μg/ml erythromycin (Em) for the selection of *lactobacilli* transformants and for the maintenance of plasmids, respectively.

Isolation of plasmid DNA and DNA manipulation. Standard methods, as described by Maniatis et al. [18], were used for recombinant DNA work. Isolation of plasmid DNA from *Lactobacilli* was carried out according to O'Sullivan and Klaenhammer [22].

Transformation of *Lactobacilli* and *E. coli*. Transformation of *L. gasseri* and *L. johnsonii* was carried out by electroporation as the method of Walker and Klaenhammer [30]. Transformation of *E. coli* was also by electroporation, or by the $CaCl_2$ treatment method [18].

Preparation of total *lactobacilli* cell extracts. The endoglucanase activity of each *lactobacillus* cell culture was estimated by dividing into two fractions of culture supernatant and whole-cell extract. The 16-h culture broth was centrifuged at 8000 *g* for 20 min at 4°C. Harvested cells were washed twice with 50 mM sodium citrate buffer (pH 6.0), suspended in the buffer, and sonicated for 20 min with the Fisher scientific sonic dismembrator Model 550. Cell debris was removed from the extract by centrifugation for 15 min at 9000 *g*.

Determination of endoglucanase activity. For the qualitative detection of endoglucanase synthesis, the congo red dye method was used [27].

Enzyme assays were carried out in 50 mM sodium citrate buffer (pH 6.0) containing 0.5% CMC. After incubation for 30 min at 60°C, the reaction mixture was stopped by boiling for 10 min. The reducing sugar produced was measured by the DNS (dinitrosalicyclic acid) reagent [19]. One unit (U) of enzyme activity was defined as that forming 1 μmole of glucose equivalent of reducing sugar per minute under the given conditions. Specific activity was defined as units per miligram of protein. Protein concentration was determined by the Bradford method [4].

Proteinases inactivation test of cellulases. The selection of small intestinal proteinase-resistant cellulase was carried out according to the method of Hall et al. [15].

Determination of plasmid stability. The segregation stability of the recombinant plasmids was investigated by the method of Bates et al. [2].

Determination of acid tolerance. A 1-ml aliquot of overnight *lactobacillus* cell culture was subjected to low-speed centrifugation at 5000 *g* for 5 min and washed two times in 0.9% NaCl. To 0.2 ml of the washed cell suspension in a 2.0-ml capacity microfuge tube was admixed 1.0 ml of pH-adjusted MRS broth (pH 2 and 3) by 1 *N* HCl and 0.3 ml of 0.5% NaCl. The materials were vortexed at setting for 10 s and incubated at 37°C in a water bath. Aliquots of 0.1 ml were removed after 0, 30, 60, and 180 min for determination of total viable count. The results were expressed as the mean and standard deviation of two determinations. Statistical analysis comprised significance testing of the difference between means using Student's *t*-test at the levels of 0.05, 0.01, and 0.001.

Determination of bile-salt tolerance. *Lactobacilli* MRS broth was prepared with 0.3 and 0.5% oxgall (Difco) dispensed in 30-ml volumes and sterilized by autoclaving at 121°C for 15 min. For each culture to be tested, one tube of each medium was inoculated with 0.3 ml of a freshly prepared overnight *lactobacilli* culture. The inoculated media were incubated at 37°C in a water bath. Growth was monitored by increases of OD_{600} with a spectrophotometer. Also, the survival ability of *lactobacilli* was demonstrated by streaking the bacteria onto solid MRS medium supplemented with 0.5% porcine bile extract (Sigma) and culturing them anaerobically at 37°C for 48 h.

Antibiotic susceptibility test. Antibiotic susceptibility patterns of *Lactobacilli* were assayed by the disc diffusion method [7] as described previously, with 11 antibiotic-impregnated paper discs (Difco). The results were expressed in terms of resistance, moderate susceptibility, or susceptibility, according to the interpretative standards [7].

Results

Construction of recombinant expression vectors. For the expression of the endoglucanase gene in *L. gasseri* and *L. johnsonii*, two recombinant plasmids were constructed with *E. coli* MC1061 as a host. A 3.2-kb *Hind*III fragment containing full-length *cel*A gene from pCT104 was introduced into promoterless *Lactobacillus–E. coli* shuttle vector pNZ123, resulting in pSD1 (Fig 1). Also, 1.4 kb *Sal*I-digested PCR fragment *cel*A gene, with its own signal sequence, was introduced into *Lactobacillus* expression vector pNZ3004, which is equipped with *Lactococcus lactis lac* A promoter, resulting in pSD2 (Fig 1).

Transformation of *Lactobacillus* strains. *L. gasseri* and *L. johnsonii* were transformed with the two expression plasmids, pSD1 and pSD2. Unfortunately, when pSD2 was electroporated into *L. johnsonii*, no transformants were obtained. The electroporation efficiency of *L. gasseri* and *L. johnsonii* was 3.2×10^2 and 4.3×10^2 CFU/μg of pSD1, respectively. That of *L. gasseri* was 7.1×10^2 CFU/μg of pSD2.

Expression of endoglucanase activity in *L. gasseri* and *L. johnsonii*. Congo red dye method [27] was applied to confirm the endoglucanase activity of transformants with

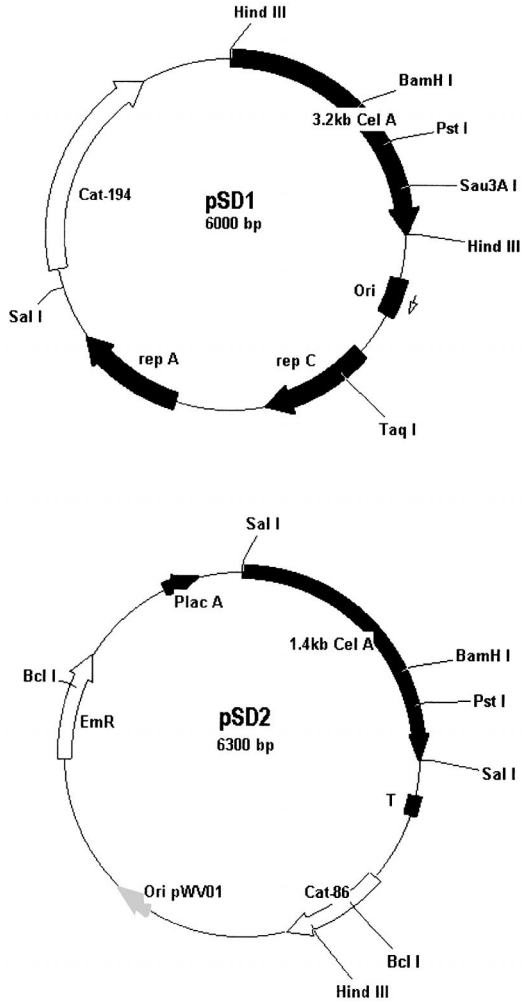

Fig. 1. *Lactobacillus* expression plasmids harboring the *C. thermocellum* cel A gene.

endoglucanase gene-carrying plasmids such as pSD1 and pSD2. When 0.1% congo red solution was poured on the surface of the MRS plates containing 0.5% CMC, yellow halo zones were formed, indicating endoglucanase activity (Fig 2).

As shown in Table 1, more than 87% of endoglucanase activity was detected in extracellular fraction of *L. gasseri* and *L. johnsonii* cells. Also, the specific activities of *L. gasseri* (pSD1), *L. gasseri* (pSD2), and *L. johnsonii* (pSD1) were 6.225, 4.056, and 5.298 U/mg of protein, respectively.

Screening for a proteinase-resistant cellulase. As shown in Table 2, the endoglucanase A from *C. thermocellum* was completely resistant to inactivation by small intestinal proteinases such as elastase, trypsin, and pancreatin.

Plasmid stability in *L. gasseri* and *L. johnsonii*. The pSD1 was maintained fairly well in *L. johnsonii* after 30 generations, as resistant colonies to antibiotics were reduced by half (Fig. 3). On the other hand, pSD1 and pSD2 were relatively stable in *L. gasseri*. Even after 40 generations, over 50% of the colonies were resistant to antibiotics in *L. gasseri* harboring pSD1 and pSD2 (Fig. 3).

Acid tolerance. The effect of low pH on the viability of *L. gasseri* and *L. johnsonii* is presented in Table 3. At pH 3, both strains survived at least 180 min, but at pH 2, *L. gasseri* reduced viability after 60 min. *L. johnsonii* was considered intrinsically more resistant to low pH than *L. gasseri*.

Bile-salt tolerance. The growth curves of *L. gasseri* and *L. johnsonii* in the presence of 0.3% and 0.5% oxgall are presented in Fig. 4. *L. gasseri* was greatly inhibited in MRS broth with oxgall, but *L. johnsonii* was relatively bile-salt resistant. Also, *L. johnsonii* showed the survival ability even in the 0.5% porcine bile extract (Fig. 5).

Antibiotic susceptibility. *L. johnsonii* and *L. gasseri* showed a rather homogeneous resistant pattern to antibiotics (Table 4). Both strains were resistant to amikacin, bacitracin, gentamicin, streptomycin, kanamycin, and colistin, while susceptible to penicillin G, amphicillin, tetracyclin, oxytetracyclin, and erythromycin.

Discussion

In this work, we were successful in the functional expression of an endoglucanase-encoding gene (*cel*A) from *C. thermocellum* in *L. gasseri* and *L. johnsonii*. *L. gasseri* and *L. johnsonii* harboring pSD1, which apparently expressed and secreted *C. thermocellum* endoglucanase under the control of its own expression and secretion signal. And *L. gasseri* harboring plasmid pSD2 also efficiently expressed and secreted endoglucanase under the control of *Lactococcus lactis lac* A promoter and its secretion signal.

This result provides further evidence for the universal nature of signal peptide recognition by Gram-positive bacteria [2], suggesting that the endoglucanase signal peptide of *C. thermocellum* be efficiently recognized by the protein transport machinery of *L. gasseri* and *L. johnsonii*. The levels of *cel*A expression in *L. gasseri* and *L. johnsonii* compared with those of cellulases expressed by other *Lactobacillus* species were relatively high. Micromole quantities of glucose equivalents per min were released by hydrolysis of CMC compared with nanomole quantities of *L. reuteri* reported previously [16].

To investigate plasmid stability in *lactobacilli*, *L. gasseri* and *L. johnsonii* harboring pSD1 and *L. gasseri* harboring pSD2 were grown in MRS medium without

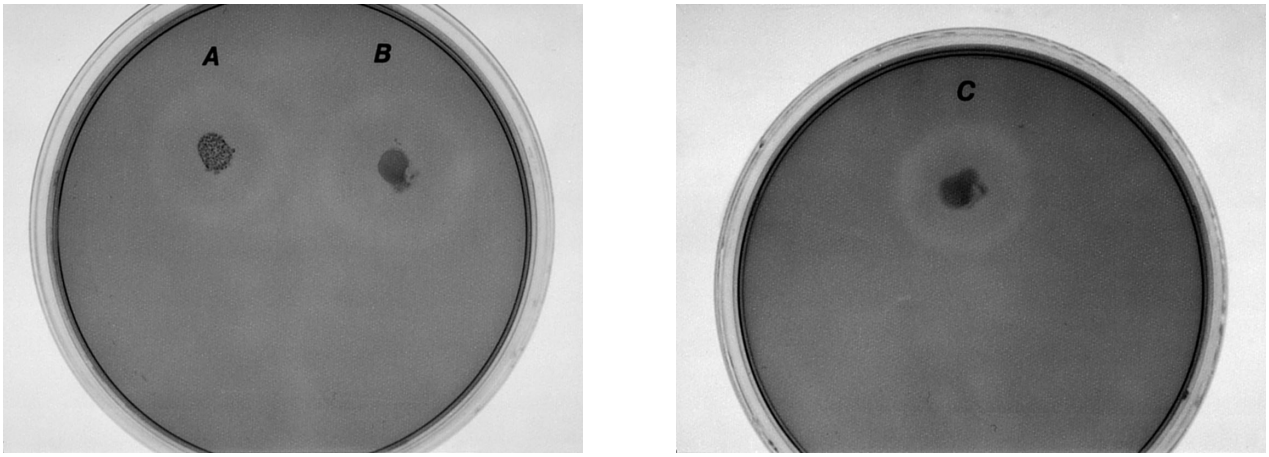

Fig. 2. Congo-red test of *Lactobacillus* transformants indicating endoglucanase activity. A: *L. johnsonii* (pSD1); B: *L. gasseri* (pSD1); C: *L. gasseri* (pSD2).

Table 1. The activity of endoglucanase in transformants of *L. gasseri* and *L. johnsonii*

Strain (plasmid)	Secretion rate (%)	Endoglucanase activity (U/ml)		
		Total	Supernatent	Whole cell extract
L. gasseri (pSD1)	98.8	0.731	0.722	0.009
L. gasseri (pSD2)	87.7	0.464	0.407	0.057
L. johnsonii (pSD1)	95.7	0.793	0.759	0.034

Table 2. Resistance of cellulases to proteolytic inactivation

Enzymes	Half life of enzyme incubated with proteinase (min)		
	Pancreatin	Trypsin	Elastase
Endoglucanase A	>120	>110	>60
Endoglucanase II	30	20	15
Endoglucanase B	60	60	60
Mixed-glucanase	10	8	8
CMC-xylanase	5	6	5

Endoglucanase A [3], II [20], and B [13] were derived from *Clostridium thermocellum*, *Actinomyces* KNG 40, and *Clostridium josui*, respectively. Mixed-glucanase [26] and CMC-Xylanase [31] were derived from *Fibrobacter succinogenes.*

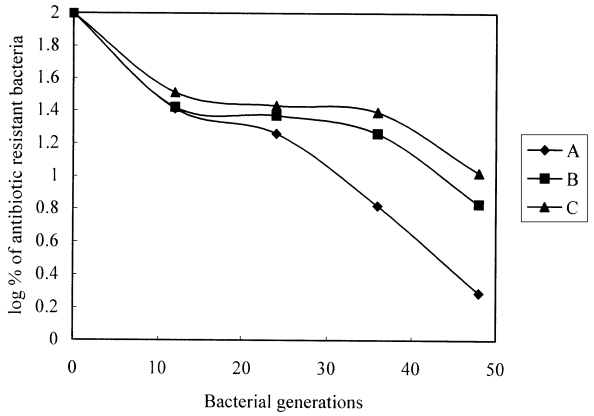

Fig. 3. Stability of plasmids in *L. gasseri* and *L. johnsonii*. A: *L. johnsonii* (pSD1); B: *L. gasseri* (pSD1); C: *L. gasseri* (pSD2).

antibiotics and maintained in mid-log phase throughout 50 generations. At appropriate times, bacteria were plated onto antibiotic-free MRS medium and MRS medium containing Cm (pSD1) and Em (pSD2) to determine the rate of plasmid loss. The results showed that pSD1 in *L. johnsonii* was more unstable than pSD1 and pSD2 in *L. gasseri*. This could be explained by the lack of tolerance of the strain to a foreign DNA or by a plasmid reorganization resulting in deletion of tolerance either at the antibiotic resistance gene or at the plasmid replication level [6].

To develop recombinant *Lactobacillus* strains with the capacity to hydrolyze cellulose in the small intestine, the secreted cellulase must not be inactivated by proteinases of pancreatic origin. Hall et al. [14] previously reported that endoglucanase E from *C. thermocellum* was completely resistant to small intestinal proteinases, and similar results were obtained in our study. Endoglucanase A from *C. thermocellum* was also most resistant to inactivation by all the small intestinal proteinases.

Lactobacillus gasseri and *L. johnsonii* were both regarded as acid tolerant. The viable counts of *L. gasseri* and *L. johnsonii* were maintained at pH 3 for at least 180 min. Acid-tolerant strains have an advantage in surviving under the low pH conditions in the stomach (pH 2.0 in extreme cases), where hydrochloric and gastric acids are secreted. Toit et al. [28] reported that *L. reuteri* BFE 1058 and *L. johnsonii* BFE 1061 isolated from the pig faeces

Table 3. Effect of low pH on viability of *L. gasseri* and *L. johnsonii*

Strain	pH	Viable count (log CFU/ml)			
		0 min	30 min	60 min	180 min
L. johnsonii	2	7.813 (0.018)	7.806 (0.012)	7.758 (0.029)	7.353 (0.054)***
	3	8.087 (0.009)	8.012 (0.056)	7.656 (0.024)***	7.577 (0.014)***
L. gasseri	2	7.417 (0.012)	7.394 (0.011)	7.208 (0.015)***	4.491 (0.014)***
	3	7.499 (0.030)	7.428 (0.017)*	7.416 (0.005)**	7.409 (0.001)**

Results are shown as mean (S.D.), n = 2. Paired sample, Student's *t*-test with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

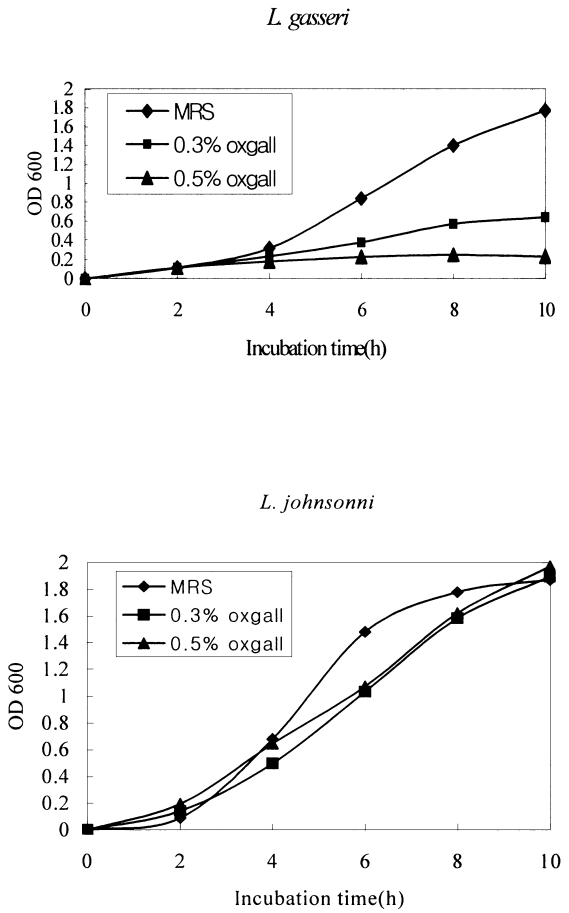

Fig. 4. Growth curve of *L. gasseri* and *L. johnsonii* with and without 0.3 and 0.5% oxgall.

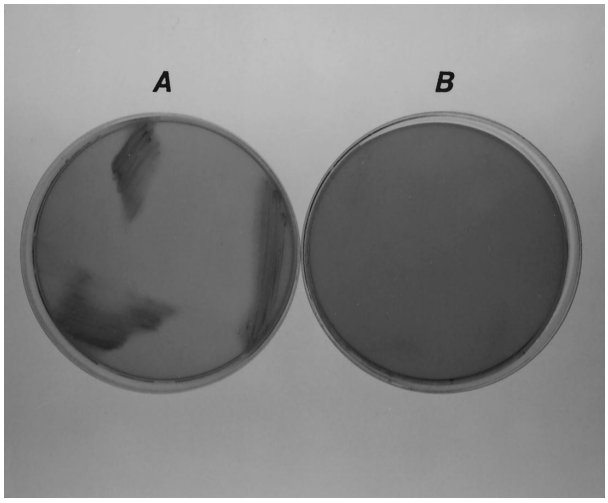

Fig. 5. The survival ability of *Lactobacilli* in the presence of 0.5% porcine bile extract. A: *L. johnsonii*; B: *L. gasseri*.

Table 4. Responses of *L. gasseri* and *L. johnsonii* to antibiotics

Antibiotics	Concentration	*L. gasseri*	*L. johnsonii*
Penicillin G	10 unit	S[a]	MS
Ampicillin	10 mcg	MS	S
Kanamycin	30 mcg	R	R
Streptomycin	10 mcg	R	R
Erythromycin	15 mcg	S	S
Amikacin	30 mcg	R	R
Tetracycline	30 unit	S	S
Bacitracin	10 unit	R	R
Colistin	10 mcg	R	R
Oxytetracycline	30 mcg	S	S
Gentamicin	10 mcg	R	R

[a] S: Susceptible, MS: Moderately susceptible, R: Resistant.

were able to grow at pH 3 and 4, and may thus be regarded as acid tolerant. Generally, the intrinsic acid resistance of *L. delbruelkii* supsp. *bulgaricus* and *S. thermophilus* is poor, whereas *L. acidophilus* and *L. bifidobacteria* have been reported to be more resistant, although great differences exist between strains [8, 9]. Most strains referred to as *L. acidophilus* in probiotic products could be identified as either *L. gasseri* or *L. johnsonii*, both members of the *L. acidophilus* group [17].

In *L. gasseri*, when compared with the control MRS broth, 0.3% oxgall exerted an inhibitory effect on bacterial growth. Also, *L. gasseri* did not survive in the presence of 0.5% porcine bile extract. Perhaps, porcine bile extract exhibited a stronger bactericidal effect than oxgall. On the other hand, *L. johnsonii* was highly bile-salt resistant, as it survived even in the presence of

0.5% oxgall and porcine bile extract. Bile salt tolerance is important for *Lactobacillus* strains to grow and survive in the upper small intestine, and non-intestinal bacteria such as *L. bulgaricus* and *L. lactis* were very sensitive to bile concentrations lower than 0.05% [14, 28].

Probiotics are often mentioned as natural substitutes to feed antibiotics, but in some cases it may be feasible to combine the probiotic and antibiotic treatments to obtain an extra advantage [21]. Dutta and Devriese [12] investigated the minimal inhibitory concentrations of some commonly used feed antimicrobial agents against *lactobacilli* isolated from pigs, cattle, and poultry. Actually, the percentages of resistant strains varied in pigs, cattle, and poultry from 2 to 70, 10 to 95, and 8 to 83, respectively, depending on drugs, and suggesting a potential for the combined treatment of antibiotics and *lactobacilli* probiotics [12]. Also, the occurrence of bacitracin-resistant *L. acidophilus* strains from cattle and poultry was related to bacitracin use as a growth promoter in these animals [7].

In conclusion, this study provides some important findings concerning a gastrointestinal species of *lactobacilli*. First, the expression of an endoglucanase gene of heterologous origin was demonstrated for the first time in *L. gasseri* and *L. johnsonii*. Second, the probiotic characteristics of these bacteria were described (for example, acid tolerance, bile-salt tolerance, antibiotic susceptibility). These results may provide useful basic information for the studies on genetic modification of gastrointestinal *lactobacilli* for biotechnological and microecological purposes.

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