



Probiotic assessment of *Enterococcus faecalis* CP58 isolated from human gut

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ABSTRACT

A total of seventy lactic acid bacteria (LAB) were isolated from the faeces of healthy humans and their identities were confirmed by sequencing of their 16S rRNA genes. Of these only 5 isolates were found to resist bile salts and indicated survival in the simulated in vitro digestion assay which reproduces the stomach and intestinal digestion indicating their tolerance to gastric enzymes and the low pH conditions. Species that showed the best resistance to these conditions were: *Lactobacillus casei*, *Lactobacillus* sp., uncultured bifidobacteria, *Enterococcus faecalis* and *Streptococcus anginosus*. These strains were investigated further to study their capacity to adhere to human intestinal Caco-2 cells. *E. faecalis* was the most adherent strain. Examination of the virulence determinants for this strain indicated that it was positive for *efA_{fs}*, *gelE*, *agg*, *cpd*, *cob*, *ccf* and *cad*, a profile that is similar to that of many *E. faecalis* isolates from food sources. The cytolysin biosynthetic genes *cylA*, *cylB* and *cylM* that are more associated with the clinical isolates of *E. faecium* were not detected in this strain. The antibiotic susceptibility tests indicated that the strain was sensitive to vancomycin, tetracycline, rifampicin and erythromycin but resistant only to kanamycin and chloramphenicol. These data suggest that the strain *E. faecalis* CP58 may be tested further for beneficial properties and developed as a new probiotic.

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1. Introduction

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit to the host (FAO/WHO, 2002). Studies that investigate beneficial properties of probiotics have been increasing in the recent years. Some of the beneficial effects of probiotics are: exclusion of pathogens (Pascual et al., 2010), alleviation of allergic response (Martínez-Cañavate et al., 2009), treatment of colitis (Resta-Lenert and Barrett, 2009) and irritable bowel syndrome (IBS) (Brenner and Chey, 2009), colon cancer prevention (Liong, 2008), immune response modulation (D'Arienzo et al., 2009; Galdeano et al., 2009) and cholesterol lowering properties (Huang and Zheng, 2009). The intestinal microbial population is a dynamic and very complex ecosystem, with an approximate number of 10¹⁴ microorganisms comprising more than 1000 species (Rajilić-Stojanović et al., 2007). Lactic acid bacteria, which are generally considered to be safe (GRAS status), are present naturally in the human intestine and for this reason they are preferentially developed for commercial use as probiotics (Gu et al., 2008; Fernandez et al., 2003). Numerous genera of bacteria and yeasts are currently used as probiotics, although the most commonly used genera are lactobacilli and bifidobacteria.

Some of the essential characteristics of a bacterial strain used as a probiotic are the ability to survive in bile salts, gastric resistance and adherence to host intestinal cells (Dunne et al., 2001).

It is important as well to take into account antibiotic resistance because probiotic strains could harbour resistance genes which may be transferred to pathogenic bacteria. In the case of enterococci which are used as dairy starter cultures and also used in fermentation of meat and vegetable products, this is particularly relevant since some enterococcal species are a cause of clinical infections and it is therefore essential to establish not only the antibiotic resistance profile but also the assessment of the virulence genes. Many virulence genes and their functions have been described in enterococci (Carlos et al., 2010). These include the *cylM*, *cyl B* and *cylA* which are involved in the posttranslational modification, transport and activation of the cytolysin respectively; *agg* encodes an aggregation protein involved in adherence to eukaryotic cells; *esp* gene responsible for a cell wall protein involved in immune evasion; *efA_{fs}* and *efA_{fm}* are adhesins expressed in serum by *Enterococcus faecalis* and *Enterococcus faecium* respectively; *cpd*, *cob*, *ccf* and *cad* are genes encoding sex pheromones that facilitate conjugation and *gelE* is responsible for the production of a toxin that hydrolyzes gelatine, collagen and other compounds. The main objective of this work was to isolate and characterise several lactic acid bacteria of human GI tract origin as potential new probiotic strains and assess their safety.

2. Materials and methods

2.1. Isolation of gut bacteria

Faecal samples used in this study were collected from 3 healthy adults (aged 20–40). Volunteers had not been prescribed antibiotics

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for at least three months prior to the study or had taken any probiotics. Faecal samples were homogenized in anaerobically reduced phosphate buffered saline (PBS) (pH 7.0) using a Stomacher 400 (Seward, UK) operating at 230 rpm for 45 s. Aliquots were serially diluted (10-fold) in pre-reduced PBS, containing peptone (5 g/L), NaCl (2.5 g/L) and L-cysteine HCl (0.5 g/L, pH 7.0), in an anaerobic cabinet. Dilutions were plated in triplicate onto selective media. For the isolation of lactic acid bacteria, three different media were used; Rogosa, LAMVAB and MRS agar (Oxoid). All media plates were preincubated at 37 °C overnight in an anaerobic cabinet (MACS-MG-1000-anaerobic workstation, DW Scientific) under an atmosphere of 5% CO₂, 10% H₂ and 85% N₂. From each selective media, colonies with different morphologies were subcultured in MRS broth and then stored at –80 °C in glycerol (40% v/v).

2.2. PCR amplification and identification of isolates

Primers for lactobacilli were those initially used by Kullen et al. (2000). The primers amplify an approximately 500 bp region of the 16S rRNA gene, which contained the V1 and V2 variable regions. The primer sequences were: plb16 5'-AGAGTTGATCCTGGCTCAG-3' and mlb16 5'-GGCTGCTGGCACGTAGTTAG-3'.

All amplification reactions were carried out in a Hybaid PCR Sprint thermocycler (Thermo Electron). The total volume of each reaction was 50 µL. PCR conditions for both primer sets were optimised for direct cell amplification. The thermocycle programme used for the PCR amplifications consisted of one cycle of 96 °C for 4 min followed by 30 cycles of 96 °C for 30 s, 48 °C for 30 s and 72 °C for 45 s, and then one cycle of 72 °C for 4 min. The PCR products were resolved by electrophoresis in a 1.2% (w/v) agarose gel (Sigma) and visualised by ethidium bromide staining. PCR amplified products were cleaned with Wizard PCR Preps DNA Purification System (Promega) according to the manufacturer's instructions and used as a template in the DNA sequencing reactions using an ABI Prism BigDye v3.1 Terminator Cycle Sequencing Ready Reaction kit. Sequences obtained were interrogated by BLAST search (Altschul et al., 1990) using the NCBI database. The identities of the isolates were determined on the basis of the highest matching score.

2.3. Survival in bile salts

The strains were grown in MRS broth. The overnight culture was adjusted to pH 6 and a solution of bile salts (Oxoid) was added to a final concentration of 0.3% (v/v). In the control tube the addition of bile salt was omitted. Samples were incubated for 6 h at 37 °C and aliquots were taken before adding bile salts and then at times 0, 1, 3 and 6 h for determination of cell count by plating in triplicate onto MRS agar. The plates were incubated under anaerobic conditions for 48 h.

2.4. Survival in simulated in vitro digestion

In vitro digestion was performed as described by Seiquer et al. (2001) with some modifications. Strains were grown in skim milk overnight. One gram of the fermented milk was diluted 1/10 in PBS. To simulate the gastric digestion, the sample was adjusted to pH 3.0 and Pepsin (Sigma) was added to a final concentration of 5% (w/v). The mix was incubated at 37 °C for 90 min with agitation at 110 rpm. To create intestinal digestion conditions the sample was adjusted to pH 6.0 and solutions of Pancreatin (Sigma) and bile salts at a final concentration of 0.1% and 0.3% (w/v) respectively were added. Samples were incubated for 150 min at 37 °C with agitation (110 rpm). Samples were removed for determination of cell count before and after both gastric and intestinal digestion. An aliquot was serially diluted and then plated on MRS agar in triplicate. The plates were incubated under anaerobic condition for 48 h.

2.5. Adhesion of bacteria to Caco-2 cells

Ability of the isolated bacteria to adhere to the human colon adenocarcinoma cell line Caco-2 was investigated. The cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma), 1% (v/v) non-essential amino acids (Sigma), and 1% (v/v) penicillin-streptomycin (Sigma). The medium was changed every 2–3 days. Cells were grown on 24-well tissue culture plates (BD Falcon®) at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air until a confluent monolayer was obtained.

Wells with a Caco-2 cell monolayer were washed once with antibiotic-free DMEM before the adhesion assay. Bacteria were diluted in DMEM to a concentration of 1×10^7 cfu/ml and added to each well in 1 ml (total volume) and incubated for 3 h at 37 °C in an atmosphere of 5% (v/v) CO₂. After incubation, each monolayer was washed three times with a sterile pre-warmed (37 °C) solution of Hanks buffered saline solution (Sigma) to remove free, non attached bacterial cells. Then 1 ml of 1% (v/v) Triton × 100 and a sterile magnetic flea was placed in each well. The suspension was stirred for 10 min to allow the cells to be detached from the well. Serial dilutions of the cell suspension were plated onto MRS plates (Oxoid) and incubated under anaerobic conditions at 37 °C to determine viable cell number. Adhesion assays were performed in triplicate.

2.6. Antibiotic susceptibility

Antibiogram for *E. faecalis* CP58 was determined using the antibiotic diffusion discs (Oxoid). CP58 was inoculated in MRS broth and incubated at 37 °C overnight. 100 µL of the diluted culture (approximate 10^7 viable cells) was streaked onto MRS agar and antibiotic discs were applied onto the surface using an antibiotic disc dispenser (Oxoid). The antibiotics tested were used at the following concentrations: 10 µg tetracycline, 10 µg chloramphenicol, 30 µg kanamycin, 15 µg erythromycin, 30 µg rifampicin and 30 µg vancomycin. Plates were incubated at 37 °C under anaerobic conditions and evaluated after 24 h inoculation. Inhibition zones around the discs were measured using a digital calliper. Results were interpreted according to the cut-off levels proposed by Charteris et al. (1998a) with strains considered resistant if inhibition zone diameters were equal to or smaller than 20 mm for rifampicin, 14 mm for vancomycin and tetracycline, and 13 mm for kanamycin, chloramphenicol and erythromycin.

2.7. Screening of *E. faecalis* for virulence determinants

Enterococcal strains were grown in BHI (Oxoid) at 37 °C. Control strains used in this study were *E. faecium* FI9226, FI9190 (*efAfs* + *gelE* + *agg* + *cylMBA* + *esp* + *cpd* + *cob* + *ccf* + *cad* +) and FI9245 (*efAfm* + *efAfs* + *esp* +). Genomic DNA was isolated from an overnight culture of *E. faecalis* using the Qiagen genomic tip system. PCR to screen for the specific virulence factors (*efAfs*, *gelE*, *cylM*, *CylB*, *cylA*, *agg2*, *espfs*, *efAfm*, *cad*, *cob*, *cpd*, *ccf*, and *espfm*) was performed exactly as described by Eaton and Gasson (2001).

3. Results

3.1. Isolation and identification of human gut bacteria

A total of 53 strains were isolated from Rogosa and LAMVAB agar. Of these 28 were *Lactobacillus casei*, 12 *Lactobacillus paracasei*, 10 *Lactobacillus* sp., 2 *Lactobacillus rhamnosus* and 1 *Lactobacillus paracasei* sbsp. *paracasei*. A total of 17 bacterial strains were isolated on MRS agar of which 9 were *E. faecalis*, 5 were *Streptococcus anginosus*, 2 uncultured *bifidobacteria* and 1 *Enterococcus vaginalis*

(Table 1). The fact that the primers used were able to amplify lactic acid bacteria other than lactobacilli indicates that these primers were not as specific as expected.

Table 1
Screening of lactic acid bacteria for their resistance to bile salts (0.3% v/v).

Strain code	Species	Bile salts survival (%)
CP01	<i>Lactobacillus casei</i>	87.5
CP02	<i>Lactobacillus casei</i>	25.9
CP03	<i>Lactobacillus paracasei/L. casei</i>	29.1
CP04	<i>Lactobacillus casei</i>	24.3
CP05	<i>Lactobacillus</i> sp.	88.4
CP06	<i>Lactobacillus casei</i>	38.7
CP07	<i>Lactobacillus casei</i>	0
CP08	<i>Lactobacillus paracasei/L. casei</i>	0
CP09	<i>Lactobacillus paracasei/L. casei</i>	0
CP10	<i>Lactobacillus paracasei/L. casei</i>	0
CP11	<i>Lactobacillus paracasei/L. casei</i>	0
CP12	<i>Lactobacillus paracasei/L. casei</i>	0
CP13	<i>Lactobacillus casei</i>	0
CP14	<i>Lactobacillus casei</i>	0
CP15	<i>Lactobacillus casei</i>	0
CP16	<i>Lactobacillus casei</i>	0
CP17	<i>Lactobacillus casei</i>	0
CP18	<i>Lactobacillus paracasei/L. casei</i>	0
CP19	<i>Lactobacillus</i> sp.	0
CP20	<i>Lactobacillus casei</i>	0
CP21	<i>Lactobacillus paracasei/L. casei</i>	48.6
CP22	<i>Lactobacillus casei</i>	51.8
CP23	<i>Lactobacillus casei</i>	0
CP24	<i>Lactobacillus</i> sp.	27.6
CP25	<i>Lactobacillus casei</i>	0
CP26	<i>Lactobacillus casei</i>	52.7
CP27	<i>Lactobacillus casei</i>	33.9
CP28	<i>Lactobacillus casei</i>	0
CP29	<i>Lactobacillus paracasei/L. casei</i>	17.4
CP30	<i>Lactobacillus</i> sp.	31.5
CP31	<i>Lactobacillus casei</i>	27.7
CP32	<i>Lactobacillus paracasei/L. casei</i>	30.2
CP33	<i>Lactobacillus paracasei/L. casei</i>	42.9
CP34	<i>Lactobacillus casei</i>	41.8
CP35	<i>Lactobacillus casei</i>	18.3
CP36	<i>Lactobacillus paracasei/L. casei</i>	0
CP37	<i>Lactobacillus casei</i>	59.9
CP38	<i>Lactobacillus casei</i>	51.9
CP39	<i>Lactobacillus</i> sp.	43.1
CP40	<i>Lactobacillus casei</i>	67.16
CP41	<i>Lactobacillus casei</i>	26.21
CP42	<i>Lactobacillus casei</i>	0
CP43	<i>Lactobacillus casei</i>	46
CP44	<i>L. rhamnosus</i>	59.6
CP45	<i>L. rhamnosus</i>	39.3
CP46	<i>Lactobacillus casei</i>	72.3
CP47	<i>Lactobacillus casei</i>	51.6
CP48	<i>L. paracasei</i> sbsp <i>paracasei</i>	18.2
CP49	<i>Lactobacillus</i> sp.	25.5
CP50	<i>Lactobacillus</i> sp.	90.1
CP51	<i>Lactobacillus</i> sp.	37.1
CP52	<i>Lactobacillus</i> sp.	0
CP53	<i>Lactobacillus</i> sp.	18.5
CP54	Uncultured bifidobacteria	92.2
CP55	Uncultured bifidobacteria	95.6
CP56	<i>Enterococcus faecalis</i>	50.6
CP57	<i>Enterococcus faecalis</i>	48.8
CP58	<i>Enterococcus faecalis</i>	92.6
CP59	<i>Enterococcus faecalis</i>	47.2
CP60	<i>Enterococcus vaginalis</i>	41.4
CP61	<i>Enterococcus faecalis</i>	39
CP62	<i>Enterococcus faecalis</i>	54.7
CP64	<i>Enterococcus faecalis</i>	49.9
CP65	<i>Streptococcus anginosus</i>	57.7
CP66	<i>Streptococcus anginosus</i>	0
CP67	<i>Streptococcus anginosus</i>	0
CP68	<i>Streptococcus anginosus</i>	78.9
CP69	<i>Streptococcus anginosus</i>	86.8
CP71	<i>Enterococcus faecalis</i>	57
CP72	<i>Enterococcus faecalis</i>	40.8

3.2. Bile salts survival

All 70 strains were incubated for 6 h in the presence of 0.3% bile salts. Of those, just 22 strains were resistant after the incubation period with survivability higher than 50%. Only 7 strains demonstrated bile salts resistance with survival at a rate greater than 80% (Table 1). The species most resistant to bile salts were strains of *L. casei*, *Lactobacillus* sp., uncultured *Bifidobacterium* sp., *E. faecalis* and *S. anginosus*.

3.3. Survival in simulated *in vitro* digestion

The ability to survive in the GI tract is one of the main desirable characteristics required for a probiotic. Some of the best strains that survived the bile salts assay were selected and tested further via simulated digestion test. This test allowed us to select 5 strains (CP01, CP05, CP40, CP44 and CP58) that had a good digestion survival phenotype and reduced the isolates for further testing (Table 2). The resistant strains were species of *Lactobacillus casei*, *Lactobacillus* sp. and *E. faecalis*.

3.4. Adhesion assay of LAB to Caco-2 cells

The above 5 digestion-resistant strains were examined further for their ability to colonise Caco-2 cells. The results (Table 2) indicated that the *E. faecalis* strain CP58 was the most adherent strain with an adhesion value of 2.6×10^5 cfu/ml.

3.5. Antibiotic susceptibility

E. faecalis CP58 was found to be sensitive to vancomycin, tetracycline, rifampicin and erythromycin but resistant to kanamycin and chloramphenicol.

3.6. Screening of *E. faecalis* for virulence determinants

Currently there are many enterococcal strains used as probiotics but care must be taken to ensure they are not pathogenic for the human host. Since the best adherent strain identified in this study was *E. faecalis*, we decided to test this strain for the presence of the known virulent factors by PCR amplification of different putative virulence genes. Of the 13 genes tested, *E. faecalis* CP58 was found to be positive for the following seven virulence determinants *efAfs*, *gelE*, *agg*, *cpd*, *cob*, *ccf* and *cad* (Table 3). The sizes of the amplification products were comparable to those obtained from *E. faecium* FI9190, which is known to harbour all the genes under analysis, with exception of the gene *efAfm* which was compared with the gene product obtained from *E. faecium* FI9245.

Table 2
Survival of bile salt resistant strains in simulated digestion condition and their capacity to adhere to Caco2 cell line.

Strain code	Species	Bile salts survival (%)	Digestion survival (%)	Adhesion to Caco2 no. of cells (cfu/mL)
CP01	<i>Lactobacillus casei</i>	87.5	27.0	4.4×10^3
CP05	<i>Lactobacillus</i> sp.	88.4	23.8	4.8×10^3
CP37	<i>Lactobacillus casei</i>	59.9	30.0	3.1×10^4
CP40	<i>Lactobacillus casei</i>	67.2	24.4	3.5×10^4
CP44	<i>Lactobacillus rhamnosus</i>	59.6	0	–
CP46	<i>Lactobacillus casei</i>	72.3	0	–
CP50	<i>Lactobacillus</i> sp.	90.1	0	–
CP54	Uncultured bifidobacteria	92.2	0	–
CP55	Uncultured bifidobacteria	95.6	0	–
CP58	<i>Enterococcus faecalis</i>	92.6	42	2.6×10^5
CP69	<i>Streptococcus anginosus</i>	86.8	0	–

Table 3

Detection of virulence-related genes in enterococcal strains used in this study.

Strain	Species	Source	Virulent factors
F28 (FI9226)	<i>E. faecium</i>	Slaughterhouse broiler	None
F24 (FI9190)	<i>E. faecium</i>	Stilton cheese	<i>efafAfs</i> + <i>gelE</i> + <i>agg</i> + <i>cylMBA</i> + <i>esp</i> + <i>cpd</i> + <i>cob</i> + <i>ccf</i> + <i>cad</i> + <i>efafAfm</i> + (<i>efafAfs</i> +) <i>esp</i> + <i>efafAfs</i> + <i>gelE</i> + <i>agg</i> + <i>cpd</i> + <i>cob</i> + <i>ccf</i> + <i>cad</i> +
P11 (FI9245)	<i>E. faecium</i>	Blood	
CP 58	<i>E. faecalis</i>	Human faeces	

4. Discussion

Bacteria most commonly used as probiotic frequently belong to members of lactic acid bacteria and bifidobacteria. These microorganisms are found as commensals in the human gastrointestinal tract, have a long history of safety and have demonstrable beneficial properties. Gut microbiota from healthy volunteers could therefore be a good source of probiotics. The aim of this study was to evaluate the potential probiotic properties of a number of isolates from human GI tract.

For bacteria to be effective as a probiotic, their ability to survive the passage through the upper digestive tract to reach the large intestine where its beneficial action is expected is an essential requirement (Bezkorovainy, 2001; Charteris et al., 1998b; Marteau et al., 1997; Tuomola et al., 2001). Once they have reached the intestine, adhesion of the probiotic microorganisms to the intestinal mucosa is a prerequisite for colonisation; they must adhere to the mucus layer to avoid being removed from the colon by peristalsis. In the present study we examined a total of 70 gut isolates and of these only 5 were able to survive both the exposure to bile acids and the simulated digestion condition of the stomach with low pH environment. We further tested these strains for their adhesion to Caco-2 cells. In conclusion we found that *E. faecalis* strain CP58, was shown to be the most resistant to digestion conditions and was best at adhering to Caco-2 cells. This screening indicates that this strain may be a candidate for potential new probiotic.

A number of probiotics products currently in the market include some enterococci preparations. Examples of these are: Symbioflor 1 (SymbioPharm, Herborn, Germany), Cylactin® (Hoffmann – La Roche, Basel, Switzerland) and ECOFLOR (Waltlers Health Care, DenHaag, The Netherlands). Enterococci are also widely used as starter cultures and many of them are found in high numbers in fermented meat, dairy and vegetable products (Gomes et al., 2008; Martín-Platero et al., 2009; Ruiz-Moyano et al., 2008; Pimentel et al., 2007; Valenzuela et al., 2009) where they contribute to product ripening and flavour development. They are also present in raw fruits (Abriouel et al., 2008a) and probiotic preparations (Domann et al., 2007; Senok, 2009). However many enterococcal species are a major cause of nosocomial infections related to urinary-tract infections, bacteraemia, wound and abdominal infections (Hancock and Gilmore, 2000; Richards et al., 2000; Thurlow et al., 2009). Enterococcal as pathogens are of increased concern due to their intrinsic and acquired resistance to many antibiotics. Therefore safety assessment with regards to virulence traits and antibiotic resistance is an essential phase in the selection of enterococci as potential probiotics. We therefore examined the isolate *E. faecalis* CP58 for the presence of 13 putative virulence genes. We found that CP58 strain carries seven virulence genes (*efafAfs*, *gelE*, *agg*, *cpd*, *cob*, *ccf* and *cad*). In general *E. faecalis* strains are known to contain multiple virulence determinants, between 6 and 11. In fact this strain shares the precise virulence genotype with many of the *E. faecalis* isolates used as commercial starter or were found in raw or fermented milk products (Eaton and Gasson, 2001). Several authors have compared the virulence genes profile between clinical and food isolates (Semedo et al., 2003; Franz et al., 2001). The most important virulence trait in *E. faecalis* seems to

be the cylolysin (Kayser, 2003) which lyses the eukaryotic cells. The CP58 was negative for all the cylolysin genes *cylM*, *cylB* and *cylA* which are required for the production of active cylolysin; specifically *cylM* plays a role in posttranslational modification of the cylolysin, *cylB* is involved on the transport and *cylA* in the activation of the cylolysin. This was confirmed by other authors (Abriouel et al., 2008b) who observed that *E. faecalis* from clinical isolates had higher percentage of positives for *cylM*, *cylB* and *cylA* while some or all of them were missing in enterococcal isolates from non-clinical sources such as fruit, vegetable foods, soil and water. They also found that clinical isolates had higher rates of genes encoding for enterococcal surface protein (*esp*) and the sex pheromone (*ccf*). CP58 was negative for *esp*, but was positive for *ccf*. In comparison all the *E. faecium* isolates (food and clinical) were positive for *ccf* gene (Eaton and Gasson, 2001). A recent study (Lempäinen et al., 2005) investigated the prevalence of virulence factors among human intestinal enterococcal isolates and concluded that commensal enterococcal strains differ from the clinical isolates and that the most frequent virulence factors present in the commensal enterococci were *cpd*, *agg* and *gelE*. Our results are consistent with these findings and we also demonstrate the presence of these three genes in the strain CP58.

Positive identification of the presence of a particular virulence gene by PCR does not necessarily imply that the gene is functional and transcriptomic studies are required to establish this. The recent study by Carlos et al. (2010) indicated that expression of individual virulence genes was environmental and strain dependent. Although many of the genes present in CP58 here are classed as virulence they are not necessarily associated with pathogenesis and in fact the expression of some of these genes that encode for aggregation may be an advantageous trait that allows the bacteria to colonise the host tissue and display probiotic functionality (Domann et al., 2007).

Antibiotic resistance of enterococci is of major concern in the medical setting since antibiotic resistance genes in this organisms are often plasmid or transposon associated presenting a risk of horizontal gene transfer (Hasman et al., 2005). In general it is found that enterococcal isolates of non-clinical origin display lower incidence of antibiotic resistance compared to clinical isolates (Abriouel et al., 2008a). We determined the susceptibility of strain CP58 to several antibiotics. The strain was sensitive to clinically relevant tetracycline, rifampicin and erythromycin and more importantly to vancomycin which is the antibiotic of last resort. However this strain was found to be resistant to kanamycin and chloramphenicol. Kanamycin resistant trait was also observed in most of the enterococcal strains isolated from human and pig faeces as well as those obtained from fermented meat products (Ruiz-Moyano et al., 2009).

In summary we have identified a strain of *E. faecalis* from the human GI tract that can survive the digestive system and has the potential to colonise the intestinal epithelial cells. Furthermore we also showed that it contains several virulent genes that are commonly found in commensal gut enterococci and does not harbour the genes encoding the cylolysin which are more associated with the clinical isolates. Further work is in progress to establish if any of the virulence genes identified are expressed and to test for probiotic functionality.

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The impact of storage buffer, DNA extraction method, and polymerase on microbial analysis

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Next-generation sequencing approaches used to characterize microbial communities are subject to technical caveats that can lead to major distortion of acquired data. Determining the optimal sample handling protocol is essential to minimize the bias for different sample types. Using a mock community composed of 22 bacterial strains of even concentration, we studied a combination of handling conditions to determine the optimal conditions for swab material. Examining a combination of effects simulates the reality of handling environmental samples and may thus provide a better foundation for the standardization of protocols. We found that the choice of storage buffer and extraction kit affects the detected bacterial composition, while different 16S rRNA amplification methods only had a minor effect. All bacterial genera present in the mock community were identified with minimal levels of contamination independent of the choice of sample processing. Despite this, the observed bacterial profile for all tested conditions were significantly different from the expected abundance. This highlights the need for proper validation and standardization for each sample type using a mock community and blank control samples, to assess the bias in the protocol and reduce variation across the datasets.

Microorganisms colonize various anatomical sites and play a crucial role in the balance of health and disease. The vaginal microbiome is known to maintain the health of women and thereby prevents urogenital diseases¹. The advent of cultivation-independent molecular approaches, such as 16S rRNA amplicon sequencing, has allowed for a better understanding of the microbes that inhabit different biological niches. However, these powerful tools are not without important technical caveats that can lead to a distortion in the acquired data². Such limitations have been well documented, and include sample collection, storage buffer, DNA extraction, amplification primers and methods, sequencing technology, and analysis techniques^{3,4}. While it is impossible to negate all of these influences, it is important to understand the bias inherent in the analysis. Studies focusing on one or two technical limitations have made recommendations for improving the bias such as reducing the number of PCR cycles⁵ or adding additional lysis pre-treatment⁶.

DNA extraction, a critical step in culture-independent bacterial profiling, has been identified as a key driver of technical variation³. Most common studies on the microbiome of swab material use commercially available DNA extraction kits that vary in their lysis approach from mechanical to enzymatic treatment. Various studies have focused on technical variations in extraction kits, yet a field-wide consensus on sample extraction has not been reached^{3,6–9}. Due to the large variety of microbiota and sample types, a single standard for all sample types is unlikely to be achieved. Despite the knowledge that the choice of extraction kit can have a significant effect on the results, there is often a lack of proper validation across sample types³.

Similar to DNA extraction kits, the choice of sample storage buffer has been shown to influence the detected bacterial community^{10–12}. The ideal storage choice largely depends on the available resources during sampling such as the availability of freezing conditions¹¹. Selecting the optimal storage buffers is dependent upon its compatibility with all downstream analyses including the extraction method. Many studies, however, only focus on the effect of a single technical variation instead of examining the effect of different combination of storage buffer, DNA extraction kit, and amplification methods². Studying a combination of effects mirrors the reality of sample handling more closely and may thus provide a better foundation for the standardization of sampling handling protocols prior to microbial analysis.

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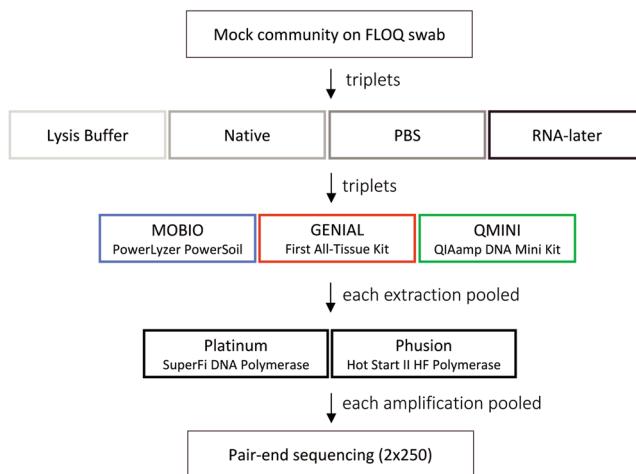


Figure 1. Outline of experimental design. A schematic showing the different treatment variables.

Extraction Method	Abbreviation	Lot #	Lysis type	Elution Volume
MOBIO PowerLyzer PowerSoil Kit	MOBIO	PL16C30	Mechanical, Column-based	100
GEN-IAL First All-Tissue Kit	GENIAL	0091.01	Enzymatic, Phenol-Chloroform	20
QIAamp DNA Mini Kit	QMINI	154035749	Enzymatic, Column-based	70

Table 1. Commercial extraction kits used in this study.

In this study, we used a mock community, composed of an even concentration of cells from 22 bacterial strains (19 genera), to assess the effect of storage buffers, extraction kits, and amplification methods (Fig. 1). Using a mock community to examine the effect of different sample handling conditions rather than environmental samples of unknown microbe composition is essential to be able to systematically compare the effects³. In addition to the use of a mock community, a blank control was included in all sample procedures to monitor any buffer, kit, or reagent specific contamination¹³. The aim of this study was to evaluate the performance of combinations of handling conditions commonly used in microbiome studies and to contribute to the ongoing debate on standardization in microbiome research.

Methods

Preparation of swab mock community samples. A cell mixture of 22 different bacterial strains at a concentration of 1×10^8 cells/mL of each organism (Microbial mock community, HM-280) in phosphate buffer saline (PBS) was obtained through Biodefense and Emerging Infectious Research (BEI) Resources, NIAID, NIH as part of the Human Microbiome Project (Manassas, USA; Supplementary Table S1). To simulate physiological conditions, 10 μ L of mock community containing 1×10^6 cells/mL of each organism was added to a flocked swab (FLOQSwabs, Copan Improve Diagnostics, Brescia, Italy) and then placed in 500 μ L of the respective storage buffer (Fig. 1). Four different storage buffers were used; PBS (PAN-Biotec GmbH, Aidenbach, Germany), a custom-made lysis buffer (10 mM Tris, pH 8.0, 0.1 M EDTA, pH 8.0 and 0.5% SDS), RNA-later (Thermo Fisher Scientific Inc., Waltham, MA, USA), and no buffer (native). A blank control swab sample was placed in each storage buffer without additive. All swab samples were frozen at -80°C for one week prior to DNA extraction. Suitable precautions were taken during sample handling and processing to insure sterility during all procedures.

DNA Extraction methods. Three commercially available DNA extraction kits were used in this study to extract bacterial DNA from swab material stored in four different storage buffers (Table 1). Extraction was performed in triplets and the extracted DNA from each buffer was subsequently pooled prior to 16S rRNA gene amplification. Processing of swab samples prior to DNA extraction is illustrated in Supplementary Fig. S1.

QIAamp DNA Mini Kit (QMINI). Samples were extracted using the QIAamp Mini Kit (Qiagen GmbH, Hilden, Germany) according to the standard protocol with minor modifications. Briefly, proteinase K (20 mg/ μ L) was added and the samples were incubated for 50 minutes at 56°C . Then, AL buffer (Qiagen GmbH) and ethanol were added in the appropriate amount. The DNA from the lysate was subsequently purified using the spin columns provided by the manufacturer and eluted in 70 μ L AVE buffer (Qiagen GmbH).

MOBIO PowerLyzer PowerSoil Kit (MOBIO). A maximum of 750 μ L of swab lysate was added to the 0.1 mm PowerLyzer® Glass Bead Tube (Qiagen GmbH). DNA extraction was continued from step 2 as described in the MOBIO PowerLyzer PowerSoil Kit protocol (Qiagen GmbH). The DNA was eluted in a final volume of 100 μ L of Solution C6 provided in the kit.

GEN-IAL First All-Tissue Kit (GENIAL). The first All-Tissue Kit (GEN-IAL, Troisdorf, Germany) was applied according to the manufacturer's protocol with minor modifications. Briefly, 5 µl proteinase K and 5 µl dithiothreitol (DTT) was added to the lysate and incubated at 65 °C for 60 min at 600 rpm in a thermomixer (Eppendorf, Hamburg, Germany). The lysate was purified according to the standard protocol and the DNA pellet was resuspended in 20 µl of C6 buffer (Qiagen GmbH).

16S rRNA gene amplification. For each pooled extraction, the V4 region of the 16S ribosomal RNA (16S rRNA) gene was amplified in triplets using the universal primers 515F and 806R adapted with linker regions and barcoded sequences used for dual-indexing¹⁴. Platinum SuperFi DNA Polymerase (Thermo Fisher Scientific) and the Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific) were both tested for amplification. Each PCR reaction consisted of 12.5 µl of 2x PCR master mix, 6 µl of Microbial DNA-Free water (Qiagen GmbH), 1.25 µl of each primer (0.5 mM each, Metabion, Martinsried, Germany) and 4 µl of template in a total reaction volume of 25 µl. PCR cycling conditions comprised of a pre-denaturation step of 30 s at 98 °C, followed by 30 cycles of 98 °C for 10 s, 55 °C for 15 s and 72 °C for 60 s, and a final 10 min extension step at 72 °C. For a selection of four samples, five additional cycles were added to the amplification procedure to examine if additional cycles may be favorable for samples with low concentrations. The amplicon triplets were pooled, purified using 0.7x AMPure XP beads (Beckman Coulter, Brea, USA) and quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Amplicon integrity was verified for a representative number of 11 samples using a BioAnalyzer 2000 (Agilent, Palo Alto, USA) prior to pooling equimolar amounts (10 nM) of each amplicon for sequencing. For the blank samples, the maximum volume (5 µl) of sample was added to the library, as the concentrations prior to sequencing were below 10 nM. Illumina MiSeq. 2 × 250 bp paired-end sequencing (Illumina V2 chemistry) was performed in the Transcriptome and Genome Analysis Laboratory at the University of Göttingen¹⁴. All generated read files analyzed in this study were uploaded to the NCBI Sequence Read Archive (SRA) (SRP125723).

Mock community data processing and analysis. The sequencing reads were processed using the mothur software package (v.1.36.1)¹⁵. According to the MiSeq SOP¹⁴, contigs were assembled, sequences trimmed, identical sequences merged, and chimeras removed (UCHIME¹⁶). Subsequently, sequences were aligned to the SILVA bacterial reference database¹⁷. Non-bacterial sequences, cross-sample singlettons, and poorly aligned sequences were removed. The seq.error command was run for each mock sample in mothur and subsequently averaged to determine the error rate of the run. Due to low read numbers, blank control sample reads (control swabs containing no mock community) were removed from the dataset and analyzed separately. As subsampling is currently still an accepted method of normalization in microbial ecology¹⁸, the reads of the remaining mock community samples were rarefied to 95,870 sequences/sample. A separate file with the theoretical sequence composition (actual) of the 22 bacterial strains of mock community was created and adjusted for the 16S rRNA copy number (Supplementary Table S1) and normalized to the sequence count of the run (95,870 reads)¹⁹. After merging the actual (theoretical) mock community composition with the practically obtained sequences, the merged file was classified using the Bayesian classifier implemented in mothur²⁰. Operational taxonomic units (OTUs) were assigned based on 97% sequence similarity and subsequently the alpha and beta diversity was analyzed. For alpha diversity, the richness (OTUs observed and Chao1) and community diversity (Inverse Simpson Metric) was analyzed using the summary.single command in mothur. Additionally, the percentage of contaminant OTUs (OTUs that do not cluster to the theoretical mock community) was examined. Beta diversity was analyzed using Bray-Curtis dissimilarity index²¹. The dissimilarity matrix was visualized using nonmetric multidimensional scaling (NMDS) plots and Newick formatted dendrograms (visualized in FigTree v.1.4.2, <http://tree.bio.ed.ac.uk/software/figtree/>).

Statistical comparison of sequence data. To evaluate and compare the type of extraction and amplification method used, the values of the alpha or beta diversity measurement were pooled for each variable (e.g. the buffer type). The statistical significance of the pooled data was analyzed in GraphPad Prism 6 (GraphPad software, La Jolla, CA, USA). In case of normal distribution (Kolmogorov-Smirnov normality test), the parametric paired two-tailed students t-test was used for comparison. In all other cases the non-parametric Wilcoxon matched-pairs signed rank test was used. For multiple comparisons, a one-way ANOVA with Bonferroni's multiple comparisons test was applied. Differences in community structure between storage buffers and extraction methods were tested using analysis of molecular variance (AMOVA) in mothur²². Non-metric multidimensional scaling (NMDS) plot of Bray-Curtis dissimilarities and UPGMA-clustered dendrograms (Bray-Curtis) were used to visualize data points. Parsimony (mothur) hypothesis testing was performed to test whether the differential clustering of the PBS samples in the dendrograms was significant²³. Differences in the 30 most abundant OTUs were assessed using the metastats command in mothur²⁴ and p-values for differences in individual OTUs were corrected for multiple comparisons using Bonferroni correction. Values of $p < 0.05$ were considered statistically significant.

Results

The pooled library ($n = 28$ mock samples, $n = 36$ blank/control samples) produced 12,968,125 16S rRNA sequence reads, of which 9,920,805 reads were retained after quality control (77%). A total of 8,974,393 sequences, with a mean read count of 249,288 reads per sample, were retained after the sequences corresponding to the blank control samples were removed. After rarefying to 95,870 sequences per sample, *de novo* OTU picking returned 228 OTUs, of which 19 OTUs corresponding to the mock community make up more than 99% of the pooled community. The average error rate of the run was found to be 0.040% (± 0.004).

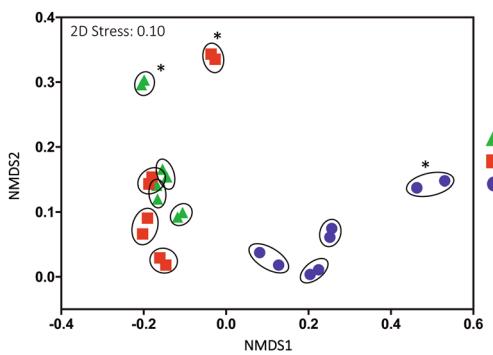


Figure 2. Clustering of samples amplified with two different polymerases on a non-metric multidimensional scaling (NMDS) plot of the Bray-Curtis dissimilarities. Points are colored by applied extraction kit. The encircled pairs correspond to a single sample where each data point represents one 16S rRNA amplification with Phusion Hot Start II High-Fidelity DNA Polymerase and the another with Platinum SuperFi DNA Polymerase. Sample pairs labeled with * were stored in PBS.

Effect of different amplification method. The choice of polymerase (Platinum SuperFi DNA polymerase vs. Phusion Hot Start II High-Fidelity DNA polymerase) was not found to significantly change the number of observed OTUs ($p = 0.08$ [paired t-test] or Inverse Simpson index, $p = 0.48$, [paired t-test]). Furthermore, pairwise comparison of the Bray-Curtis dissimilarity between the two polymerases yielded only small variations (maximum difference 0.076, Supplementary Table S2) indicating near identical bacterial community profile for a single sample (Fig. 2). Since the results indicate that these two applied high-fidelity polymerases do not significantly impact the observed microbial diversity, we pooled the data from the two polymerases for identical sample for the analyses of buffer and extraction kit choice. The addition of five cycles in 16S rRNA gene amplification shows only a minor impact on the detected bacterial composition when tested on MOBIO extractions (Supplementary Fig. S2a). There was, however, a significant increase of the number of OTUs detected with additional cycles ($p = 0.029$, Supplementary Fig. S2b), indicating that lower cycle numbers are favorable.

Effect of storage buffer. The effect of the four storage buffer (lysis buffer, native, PBS or RNA-later) on the alpha diversity was assessed based on OTU richness (identified absolute number of taxa) and evenness (Inverse Simpson index). The choice of storage buffer had no significant influence on the OTU richness of the swab samples ($p = 0.158$ [ANOVA], Fig. 3a), nor the overall evenness. However, PBS treated samples that were extracted with MOBIO, detected a lower evenness compared to all other treatment conditions (Wilcoxon test, Fig. 3b).

Pairwise AMOVA of Bray-Curtis dissimilarity showed that the storage buffer choice had a significant impact on the community structure ($p = 0.004$, AMOVA). A dendrogram of the Bray-Curtis dissimilarity shows that the PBS stored samples clustered separately from the other buffer types which was confirmed by parsimony analysis ($p = 0.001$, Fig. 3c). To examine which OTUs drive the differential clustering, we examined the read count for each OTU. Four bacterial OTUs corresponding to *Neisseria*, *Pseudomonas*, *Porphyromonas* and *Helicobacter* are significantly different in the PBS stored samples for all extraction kits (Fig. 3d–g). These results indicated that PBS buffer significantly alters single OTUs as well as the overall bacterial composition compared to all other storage buffers, independent of extraction kit choice. The bacterial profile of the blank control samples indicated that this effect is not caused by a buffer specific contamination as there appears to be no obvious buffer or kit specific profile (Supplementary Fig. S3).

Effect of extraction method. Richness, both the observed number of OTUs and Chao1, were analyzed to see the effect of the extraction kit choice on the alpha diversity. Pairwise comparison showed no significant effect on OTU richness between the different extraction kits ($p = 0.893$ [ANOVA], Table 2). In general, all extraction kits detect a higher OTU richness compared to the expected richness of the mock community (Table 2). In addition to assessing richness, evenness was analyzed using the Inverse Simpson index. The evenness of the samples extracted using MOBIO was significantly lower compared to the QMINI and GEN-IAL extractions ($p = 0.008$, $p = 0.023$, Wilcoxon test, Table 2). The evenness did not significantly vary between QMINI and GEN-IAL. Yet, the mean (\pm SEM) observed evenness (5.21 ± 0.08) was significantly lower than the expected evenness of the mock community (18.3). The same five OTUs, *Enterococcus*, *Neisseria*, *Escherichia*, *Pseudomonas*, and *Bacillus* dominate the bacterial profile independent of extraction kit choice (Fig. 3c).

Pairwise AMOVA of Bray-Curtis dissimilarity indicated that the extraction kit choice significantly impacted the community structure ($p = 0.001$, AMOVA). To assess which extraction kit more accurately represents the bacterial community structure, a theoretical ideal mock community (actual) composition was created for comparison (see methods for details). In the ideal scenario, the experimental data would be identical to the actual composition and there would be no Bray-Curtis dissimilarity. To assess the extraction kits, Bray-Curtis dissimilarity was calculated between the observed and actual mock community for each sample (Fig. 4). The samples extracted with the same commercial kit were grouped in a boxplot and pairwise comparison was performed. The QMINI kit produced a significantly better representation of the bacterial community compared to all other kits tested (paired t-test, all $p < 0.01$, Fig. 4). On the contrary, the MOBIO kit performed significantly poorer than all

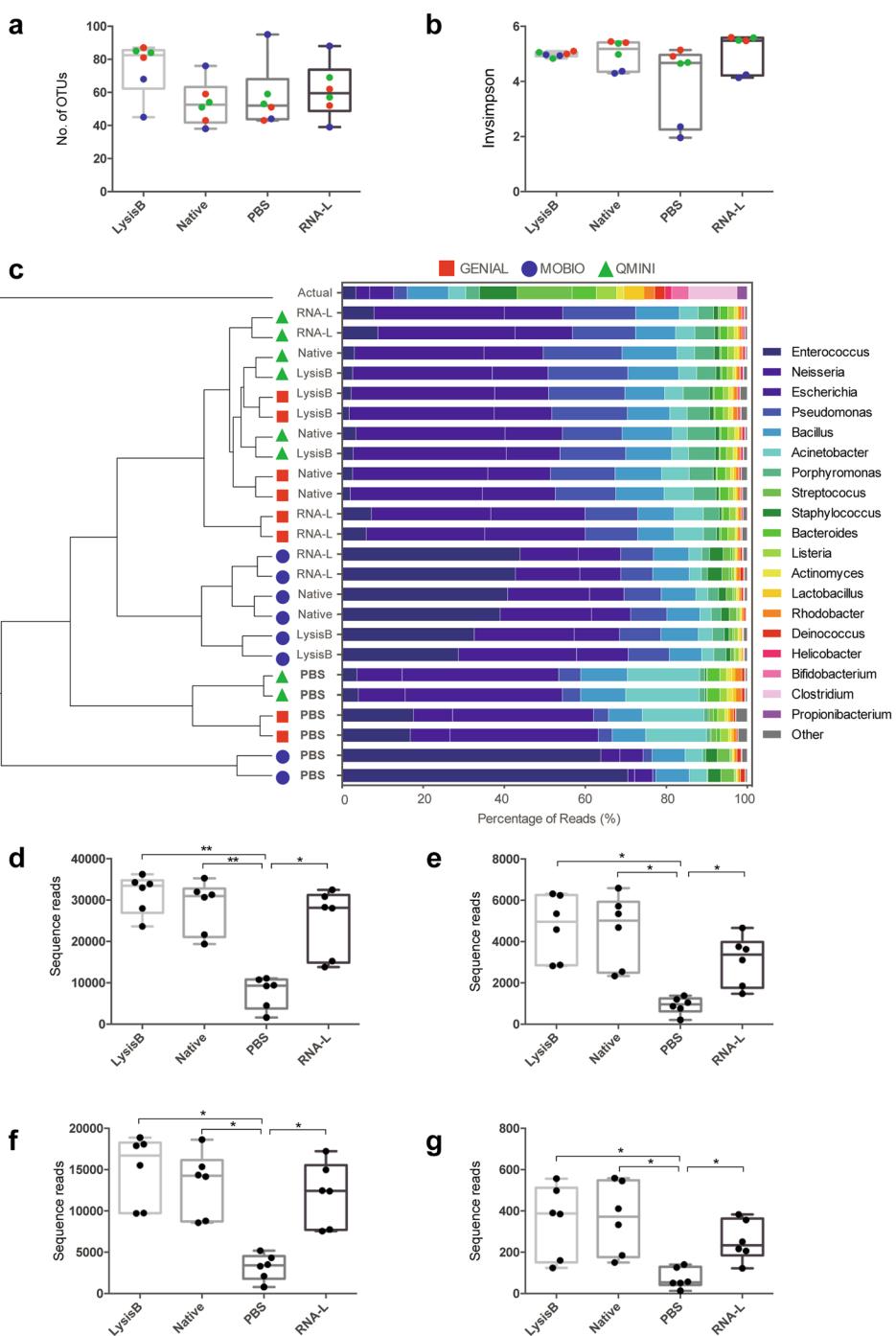


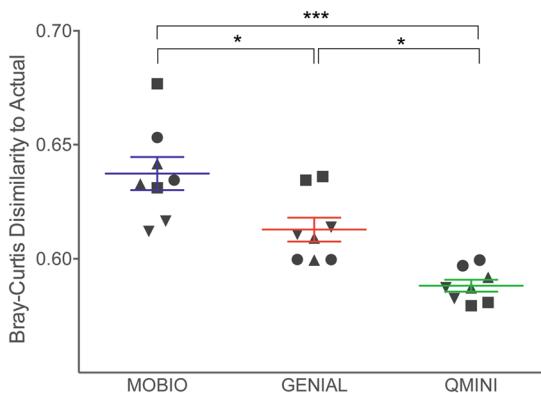
Figure 3. PBS stored samples significantly distort individual OTUs and cluster separately from other buffer types. Boxplots (median \pm range) of (a) the number of OTUs and (b) the Inverse of the Simpson index for each buffer type. (c) UPGMA clustering on Bray-Curtis dissimilarities including taxa plots showing the relative abundance of OTUs in percentage of reads. Differential clustering of PBS to all other buffers was found to be significant (parsimony test, $p = 0.001$) (d–g) Individual bacterial OTUs are significantly underrepresented for PBS-stored samples. Number of sequence reads for OTUs corresponding to (d) *Neisseria*, (e) *Pseudomonas*, (f) *Porphyromonas*, and (g) *Helicobacter*. (Wilcoxon test, * $p < 0.05$, ** $p < 0.01$).

other tested kits (all $p < 0.01$, Fig. 4). Overall, all the extraction kits distort the bacterial profile compared to the expected bacterial composition of the mock community (Fig. 4).

Discussion

We compared a variety of storage buffers, extraction kits, and amplification methods to examine which combination of handling conditions best represents the microbial diversity of an even mock community (Fig. 1). Different combinations of factors that most closely resemble the reality of sample handling were analyzed to facilitate the

Extraction Method	Observed OTUs	Chao1	InvSimpson
MOBIO PowerLyzer PowerSoil Kit	62.88 ± 8.38	69.79 ± 10.31	3.9 ± 0.40
GEN-IAL First All-Tissue Kit	59.75 ± 5.82	66.02 ± 7.51	5.3 ± 0.09
QIAamp DNA Mini Kit	64.00 ± 4.87	78.04 ± 5.94	5.1 ± 0.13
Actual/Expected Mock Community	22	22	18.3

Table 2. Alpha diversity measurements (mean ± SEM) for each of the DNA extraction kits (n = 8).**Figure 4.** Bray-Curtis dissimilarity between observed and expected strain proportion for each of the tested extraction methods. The expected strain proportion (actual) was generated for comparison and represents the theoretically composition of the mock community (see methods for detail). The pair-wise proportions (expected to observed) from samples extracted with the same commercial kit were grouped in a single boxplot (mean ± SEM). Symbols illustrate different buffer types (■ PBS, ▼RNA-later, ▲native, ●lysis buffer) (Paired t-test, *p < 0.05, ***p < 0.001).

establishment of standards for the analyses of microbial compositions in swab samples. We show that the choice of storage buffer and extraction kit affects the detected bacterial composition, while different amplification methods had only a minor effect.

Using a mock community, four storage buffers were tested that have been previously used in various studies^{25–28}. All samples in this study were frozen at -80°C rapidly after collection. The samples stored in RNA-later, lysis buffer and native performed similarly to each other and revealed a similar detected bacterial diversity (Fig. 3). Samples stored in RNA-later have been previously reported to decrease DNA purity, lower DNA extraction yields, and to significantly alter the microbial diversity compared to native frozen samples^{10,29}. This, however, was not observed in our study. It is likely, that this reflects differences in the sample material (microbes on swab vs. fecal samples) as it has been observed that fecal samples are harder to disperse evenly in RNA-later which may affect the storage and extraction efficiency¹⁰. Interestingly, compared to the other buffer types, swabs stored in PBS show an altered bacterial composition. There is no indication of a PBS buffer specific contamination profile in the blank samples that could explain this differential clustering. Moreover, PBS buffer in combination with the MOBIO extraction kit detected a lower evenness, which indicates that PBS seems to be particularly incompatible with certain extraction kits. PBS is a balanced salt solution that maintains pH, osmotic balance and is therefore frequently used as a wash buffer in cell and tissue culture. PBS storage has been recommended by manufacturers protocols and has been previously used when examining various extraction kits^{12,30}. Other studies examining the effect of different storage conditions have not tested PBS despite its use in DNA extraction from swab material^{16,10–12}. It is not clear what properties of PBS effect the mock community differently from other storage buffers. Due to its properties, the buffer may stabilize certain cell types and therefore create a different bacterial profile. Interestingly, despite the different bacterial profile, the PBS samples perform similarly to the other buffer types when comparing them to the mock community. This indicates that the choice of buffer can affect the bacterial profile and specific OTUs, but does not lead to a significantly worse representation of the bacterial community. Our findings support the notion that standardization in sample collection and handling is essential to allow comparison of data within a study³¹. Additionally, field-wide standardization across handling protocols is vital for each sample type, so that cross-study comparisons become possible.

All extraction methods used in this study identify all 19 OTUs present in the mock microbial community (22 bacterial strains of 19 genera, Supplement Table S1). However, all kits detected a higher richness compared to the actual richness of the mock control. A low concentration of mock community (approximately 1×10^7 cells/mL of each organism) was used in this study to simulate the expected bacterial amount in vaginal or oral swab samples³². Therefore, it was not surprising that additional OTUs were detected¹³. However, 99% of the pooled library clusters into 19 OTUs which correspond to the bacteria in the mock community. This indicates that the additionally detected OTUs correspond to a small fraction of sequence reads and may therefore be a result of contamination. This study in combination with previous work suggests that the expected biomass of

vaginal and oral swab samples is sufficient for amplicon-based microbial detection without the need of additional target enrichment¹³. The use of a mock microbial community in this study allowed for direct assessment of the extraction kit performance. This comparison indicated that QMINI provides the best representation of the bacterial community when compared to MOBIO and GENIAL. Using a mock community, Yuan *et al.* also found that an altered version of QMINI provided the best bacterial profile⁶. A study using oral swabs confirmed that QMINI extracts DNA with significantly greater yield and good quality compared to other extraction kits². This is in contrast to previous studies on fecal and soil samples, which found that MOBIO most effectively extracts microbial DNA of various bacterial strains³³. These reported differences in optimal extraction kit may be due to the differences in sample type. The overall bacterial DNA and exogenous material (e.g. fiber) differs substantially between fecal and swab material³⁴. Standardization of the extraction kit may thus only be appropriate within each sample type.

In this study, we find that the choice between the two polymerases and the addition of five cycles in amplification of the 16S rRNA gene did not have a significant effect on the bacterial community structure (Fig. 2). Contrary to our findings, Wu *et al.* report that the choice of polymerase had an effect on the microbial community structure, however, the two polymerases that were tested had considerable differences in the fidelity (20 times and 4 times higher than *Taq*)³⁵. The two hot-start polymerases used in our study, had significantly higher fidelity (100 times and 52 times higher compared to *Taq*) and are both recommended for NGS applications by the manufacturers. This may likely explain the lack of observable differences. Unlike polymerase choice, which had no effect on the detected evenness or richness, the addition of five PCR cycles to the amplification method led to an overestimation of the bacterial richness. Previous studies have already suggested that this increase is due to an upsurge of chimeric structures with increased cycle numbers^{3,5,35}. This supports the notion that lower cycles numbers are favorable for amplicon sequencing⁵.

All tested conditions in this study lead to a distortion of the bacterial community structure compared to the expected bacterial mock composition (Fig. 4). *Enterococcus*, *Neisseria*, *Escherichia*, and *Pseudomonas* dominated the detected profile in our study, while other bacteria genera such as *Lactobacillus* were underrepresented. Knowledge of which genera are underestimated in the detected bacterial profile (e.g. *Lactobacillus*) is essential to properly estimate the bias when studying certain bacterial communities (e.g., the vaginal microbiome). In a recent study using the same mock community, the bacterial profile resembled the one detected in our study, indicating that the observed distortion is most likely not due to laboratory or kit specific contamination^{3,13}. Instead, the bias could be attributed to a variety of factors that were not examined in this study, such as differential susceptibility of bacteria to lysis⁶. To increase lysis efficiency of a broader spectrum of bacteria, enzymatic pre-treatment has been studied as a potential solution, with mixed results^{6,36,37}. Another potential cause for the observed bias is the use of primers for 16S rRNA gene amplification. Although these are universal, amplification may favour certain bacterial strains thus creating bias in the analysis^{38,39}. Shotgun metagenomics has been proposed as a solution as it negates some of the bias caused by the amplification, however, this technique does not negate all of technical caveats as storage and extraction kit choice can still have a major impact on the results^{3,40}. Continual improvement to the sample handling conditions for both amplicon sequencing and shotgun metagenomics using mock communities is therefore essential.

Conclusion

For now, investigators should standardize the sample handling methods for each sample type as consistency among sample collection, sample storage and sample processing is able to significantly reduce variation. Preliminary tests on specific sample types should be used to ensure that the comparative analysis is as accurate as possible. Caution is, however, warranted when drawing conclusions about the relative abundance of bacterial populations in a single sample and when combining data for meta-analyses.

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Author Contributions

The study was designed by L.H.W., C.R., and S.K. Laboratory work was conducted at the German Primate Center and performed by L.H.W. and S.L. Data were analyzed by L.H.W. and S.K. All authors (L.H.W., S.L., C.R. and S.K.) contributed to the manuscript preparation.

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Differentiation of *Lactobacillus* Strains by Ribotyping

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Fifty-four lactobacillus strains were differentiated by ribotyping. The stability of ribotypes characteristic of four strains of lactobacilli inhabiting the digestive tract of mice was investigated. One of four isolates of *Lactobacillus delbrueckii* GT21, which had been associated with mice for 22 months, had an altered ribotype.

Members of the genus *Lactobacillus* can be detected in a variety of habitats including fermented foods, silage, and the digestive tracts of many animal species (10). *Lactobacillus* species make up the numerically dominant bacteria in gastric regions of the digestive tracts of pigs, rodents, and fowl (12). Lactobacilli colonize the animal host soon after birth (hatching), but, at least in piglets, a succession of *Lactobacillus* strains become dominant as the animal ages (15). This phenomenon, demonstrated by characterizing strains according to their plasmid content (plasmid profiling), may not be confined to piglets or even to young animals. Evidence based on the serological differentiation of *Escherichia coli* strains isolated from human feces suggests that the strain composition of the normal microflora of the digestive tract may fluctuate temporally (4). Since lactobacilli are known to influence markedly the biochemistry of the intestinal tract (6, 7, 14), can act as donors and recipients of plasmids encoding antibiotic resistance (5), and are promoted as being "beneficial" to the animal host (2), knowledge concerning the degree of stability of the lactobacillus microflora is essential. The study of the stability of the microflora requires a method for differentiating among *Lactobacillus* strains. The method must be applicable to all strains of lactobacilli, give consistent results, and be logically feasible in studies in which large numbers of bacterial isolates must be examined. We investigated ribotyping (9) as a means of differentiating among strains of lactobacilli, including the stability of ribotypes associated with specific strains of lactobacilli inhabiting the digestive tracts of mice.

A total of 54 strains of lactobacilli, 11 of which were from Type Culture Collections, and 1 strain of *E. coli* were examined. These strains are listed in Table 1. Total DNA (chromosomal plus plasmid) was extracted from the lactobacillus strains by the method of Flamm et al. (1) modified by Luchansky et al. (3). Total DNA was extracted from *E. coli* by the same method, except that the cells were cultured in Luria broth and mutanolysin was not required in the lysis solution.

To obtain DNA probes complementary to only 16S rDNA, we used the polymerase chain reaction to amplify the 16S rRNA gene from *Lactobacillus acidophilus* ATCC 4356 and from *E. coli* HB101. Approximately 1.5-kb fragments of 16S rDNA were amplified from total DNA from each of these two cultures by using primers modified from those of Wilson et al. (18) and Weisburg et al. (16): *SacI*-POMod (5'-CCGAGCTAACAGAGTTGATCCTGGCTC

AG-3'), the underlined sequence being a *SacI* restriction site, and *SalI*-T7-PC5 (5'-GGTCGACCGTTAATACGACTCAC TATAAGGGATACCTTGTACGACTT-3'), the underlined sequence being a *SalI* restriction site and the sequence in boldface type being the T7 promoter. The DNA was amplified by using an Autogene programmable cycling water bath (Grant Instruments Ltd., Cambridge, United Kingdom), about 100 ng of total DNA in a 100- μ l reaction mixture containing 10 μ l of 10 \times polymerase chain reaction buffer (500 mM KCl, 100 mM Tris, 15 mM MgCl₂, 40 μ g of bovine serum albumin), 40 pmol of each primer, 200 μ M (final concentration) each deoxynucleoside triphosphate, and 1.5 U of *Taq* polymerase (Amersham International, Amersham, United Kingdom). Amplification conditions consisted of one cycle of 5 min at 95°C; 30 cycles of 1 min at 93°C, 1 min at 53°C, and 3 min at 72°C; plus one additional cycle with a final 10-min chain elongation. The amplification product was cleaned with Geneclean (Bio 101, La Jolla, Calif.).

A probe containing both 16S and 23S ribosomal cDNA was synthesized by reverse transcription of *E. coli* MRE600 16S+23S rRNA (Boehringer GmbH, Mannheim, Germany) with avian myeloblastosis virus reverse transcriptase and random oligonucleotide primers. The transcription reaction mixture consisted of 1 μ g of rRNA, 2 μ l of random primers [pd(N)₆, 10 mg/ml] (Pharmacia, Uppsala, Sweden), 2 U of avian myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories Inc., Gaithersburg, Md.), 6 μ l of 5 \times reverse transcriptase buffer, 1 μ l of RNase inhibitor (2,500 U/ml; Bethesda Research Laboratories), 1 μ l of 100 mM dithiothreitol (Sigma), 2 μ l of each deoxynucleoside triphosphate at 10 mM (dATP, dGTP, and dTTP), and 1 μ l of [α -³²P]dCTP (Amersham) in a total volume of 30 μ l. The mixture was incubated for 1 h at 37°C, and the reaction was stopped by addition of 1 μ l of 0.5 M EDTA (pH 8.0). The transcription products were confirmed by electrophoresis in a 3% (wt/vol) polyacrylamide gel for 2 h at 25 V. The cDNA bands were visualized by autoradiography with X-ray film (Cronex; Dupont [Australia Ltd.], Sydney, Australia) at -70°C for 6 h.

DNA from *L. acidophilus* 706-R-7 was used to screen 16 restriction endonucleases (*Apal*, *BamHI*, *BclI*, *BglII*, *Clai*, *EcoRI*, *HindIII*, *HpaI*, *KpnI*, *NaeI*, *PstI*, *PvuII*, *SacI*, *SalI*, *SmaI*, and *XbaI*) for their suitability in preparing digests. All of the strains in the study were then tested by using four enzymes (*BclI*, *BglII*, *EcoRI*, and *HindIII*) chosen on the basis of the preliminary screening. Approximately 3 μ g of DNA was digested with the appropriate enzyme for 2.5 h as specified by the manufacturer (Boehringer GmbH). The resulting DNA fragments were separated by horizontal elec-

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TABLE 1. Bacterial strains used in this study

Strain	Source and details
<i>E. coli</i> HB101	Laboratory stock
<i>L. acidophilus</i> ATCC 4356	Appropriate culture collection
<i>L. acidophilus</i> NCTC 1899	Appropriate culture collection
<i>L. casei</i> ATCC 393	Appropriate culture collection
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> ATCC 11842	Appropriate culture collection
<i>L. fermentum</i> ATCC 14931	Appropriate culture collection
<i>L. gasseri</i> ATCC 33323	Appropriate culture collection
<i>L. helveticus</i> ATCC 15009	Appropriate culture collection
<i>L. murinus</i> CNRZ 220	Appropriate culture collection
<i>L. plantarum</i> ATCC 14917	Appropriate culture collection
<i>L. salivarius</i> subsp. <i>salicinius</i> ATCC 11742	Appropriate culture collection
<i>L. reuteri</i> DSM 20016	Appropriate culture collection
<i>L. reuteri</i> 100-23	Rodent digestive tract (17)
<i>Lactobacillus</i> sp. strain 100-14	Rodent digestive tract (8)
<i>L. acidophilus</i> 78-O-9	Porcine digestive tract (15)
<i>L. acidophilus</i> 22-16	Porcine digestive tract (15)
<i>L. acidophilus</i> 71-P-1	Porcine digestive tract (15)
<i>L. acidophilus</i> 72-P-19	Porcine digestive tract (15)
<i>L. acidophilus</i> 71-17	Porcine digestive tract (15)
<i>L. acidophilus</i> 72-R-2	Porcine digestive tract (15)
<i>L. acidophilus</i> 705-R-1	Porcine digestive tract (15)
<i>L. acidophilus</i> 706-R-7	Porcine digestive tract (15)
<i>L. fermentum</i> 78-P-4	Porcine digestive tract (15)
<i>L. fermentum</i> 71-S-12	Porcine digestive tract (15)
<i>L. fermentum</i> 22-O-6	Porcine digestive tract (15)
<i>L. fermentum</i> 22-O-4	Porcine digestive tract (15)
<i>L. fermentum</i> 22-O-5	Porcine digestive tract (15)
<i>L. fermentum</i> 71-O-10	Porcine digestive tract (15)
<i>L. fermentum</i> GT20	Laboratory stock culture (14)
<i>L. fermentum</i> isolates GT20/1 to GT20/4	Associated for 22 months with murine digestive tract
<i>L. fermentum</i> isolates GT20/5 to GT20/7	Associated for 32 months with murine digestive tract
<i>L. delbrueckii</i> 72-P-1	Porcine digestive tract (15)
<i>L. delbrueckii</i> GT18	Laboratory stock culture (14)
<i>L. delbrueckii</i> isolate GT18/1	Associated for 22 months with murine digestive tract
<i>L. delbrueckii</i> isolate GT18/2 to GT18/4	Associated for 32 months with murine digestive tract
<i>L. delbrueckii</i> GT21	Laboratory stock culture (14)
<i>L. delbrueckii</i> isolates GT21/1 to GT21/4	Associated for 22 months with murine digestive tract
<i>L. delbrueckii</i> isolates GT21/5 and GT21/6	Associated for 32 months with murine digestive tract
<i>L. reuteri</i> SH23	Laboratory stock culture (11)
<i>L. reuteri</i> isolates SH23/1 to SH23/5	Isolated from murine digestive tract 3 days, 5 days, 13 weeks (two isolates), and 16 weeks, respectively, after inoculation of animals (5)

trophoresis in a 0.8% agarose gel for 7 h at 30 mA. DNA fragments in gels were depurinated and transferred to positively charged nylon membranes (Hybond-N⁺; Amersham) by the alkali blot procedure as specified by the manufacturer, prehybridized, and then hybridized with one of three ³²P-labeled probes (16S+23S cDNA from *E. coli* MRE600, 16S rDNA from *E. coli* HB101, or 16S rDNA from *L. acidophilus* ATCC 4356) by previously described methods (5). All three probes permitted differentiation between the lactobacillus strains. Ribotypes of 22 *Lactobacillus* strains and of *E. coli* HB101 are shown as examples in Fig. 1 and 2. Identical results were obtained with the three probes, except that additional fragments were visible in autoradiographs prepared with *Hind*III-digested DNA and the *E. coli* 16S+23S probe compared with those obtained with the probes containing only 16S rDNA (Fig. 2). These additional hybridized fragments must have contained the 23S rDNA sequences of the lactobacillus chromosome because they were not observed in autoradiographs produced with *E. coli* 16S rDNA as the probe (Fig. 2). Even without the 23S sequence in the probe, all of the lactobacillus strains could be differentiated from each other on the basis of the ri-

botypes generated through the use of the four restriction enzymes (Fig. 1 and 2). Indeed, the use of two enzymes (*Eco*RI and *Hind*III) was sufficient to characterize each of the strains examined. Two strains gave identical ribotypes for all four enzymes (lanes t and v in Fig. 1, rows 1, 2, and 3, and Fig. 2, row 1). Interestingly, although obviously identical in ribotype, the strains had been identified (15) as belonging to two different species (*L. acidophilus* and *L. fermentum*), highlighting the need for improved methods for species determination of lactobacilli.

Unique ribotypes are presumably generated in bacterial strains by alterations to the sequence of nucleotide bases in DNA and therefore to the occurrence of restriction sites. If ribotyping is to be of use in differentiating lactobacilli inhabiting the digestive tract during relatively long-term studies, knowledge of the stability of the ribotypes is required. We determined the ribotypes of lactobacillus isolates of known origin that had inhabited the digestive tracts of mice for various periods and compared them with those of stock cultures (parental strains) with which the mice had been inoculated. The mice used were obtained from the various murine colonies that we maintain by gnotobiotic

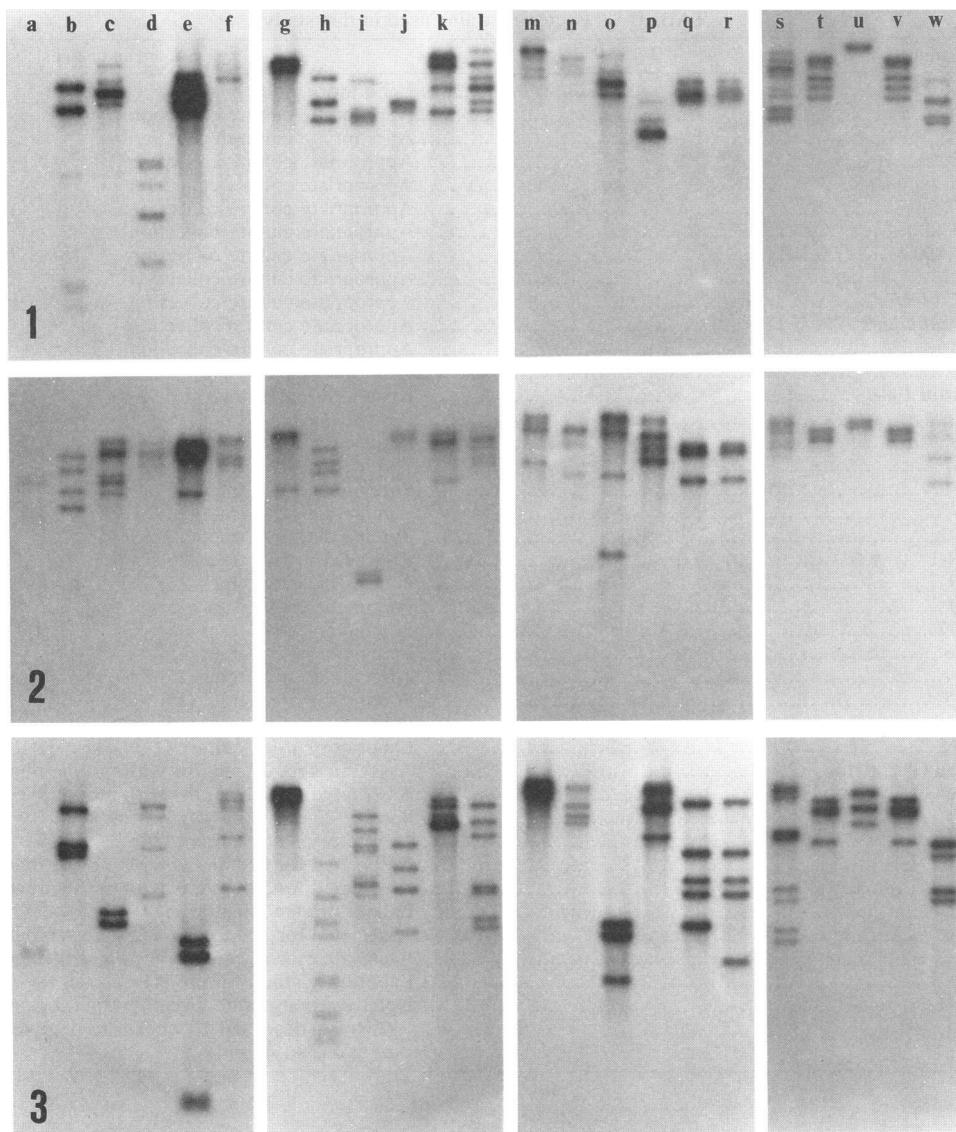


FIG. 1. Autoradiograms showing ribotypes produced by hybridization of *L. acidophilus* ATCC 4356 16S rDNA to DNA from *E. coli* and *Lactobacillus* strains. Row 1, DNA digested by *Bcl*II; row 2, DNA digested by *Bgl*II; row 3, DNA digested by *Eco*RI. Lanes (all three rows): a, *E. coli* HB101; b, *L. acidophilus* ATCC 4356; c, *L. acidophilus* NCTC 1899; d, *L. casei* ATCC 393; e, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842; f, *L. fermentum* ATCC 14931; g, *L. gasseri* ATCC 33323; h, *L. helveticus* ATCC 15009; i, *L. murinus* CNRZ 220; j, *L. plantarum* ATCC 14917; k, *L. reuteri* DSM 20016; l, *L. salivarius* subsp. *salicinii* ATCC 11742; m, *Lactobacillus* sp. strain 100-14; n, *L. reuteri* 100-23; o, *L. acidophilus* 78-O-9; p, *L. fermentum* 78-P-4; q, *L. acidophilus* 22-16; r, *L. delbrueckii* 72-P-1; s, *L. acidophilus* 71-17; t, *L. fermentum* 71-O-10; u, *L. acidophilus* 72-R-2; v, *L. acidophilus* 705-R-1; w, *L. acidophilus* 706-R-7.

technology in isolators as described elsewhere (13). Intestinal isolates of *L. reuteri* SH23 were obtained during an investigation of the in vivo transfer of plasmid pAMβ1 (5). Some of the digestive-tract isolates of *L. delbrueckii* and *L. fermentum* were from animals in our *Enterococcus*-free colony, with which the lactobacilli had been associated for 32 months (5). Other digestive-tract isolates of these strains were from our RLFL mouse colony, in which they had been microbial associates for 22 months (14). In each case, the lactobacilli had been transmitted from parents to progeny through five to six generations. Isolation methods involving medium 10, selective for lactobacilli, have been described

previously (14). An alteration in the ribotype of 1 (*L. delbrueckii* GT21/3) of 22 isolates examined was detected. The altered ribotype of *L. delbrueckii* GT21/3 was observed in digests generated by *Bcl*II, *Bgl*II, and *Eco*RI (Fig. 3A to C, lanes 4). The ribotype produced by the remaining enzyme (*Hind*III) did not differ from that of the parental strain (*L. delbrueckii* GT21; Fig. 3D, lane 4). The identity of strain GT21/3 was confirmed by biochemical testing with API50CH strips (API System, La Balme Les Grottes, France), by the gross appearance of restriction endonuclease digests run in an agarose electrophoretic gel, and by determination of its plasmid profile by the method of Tannock et al. (15). These

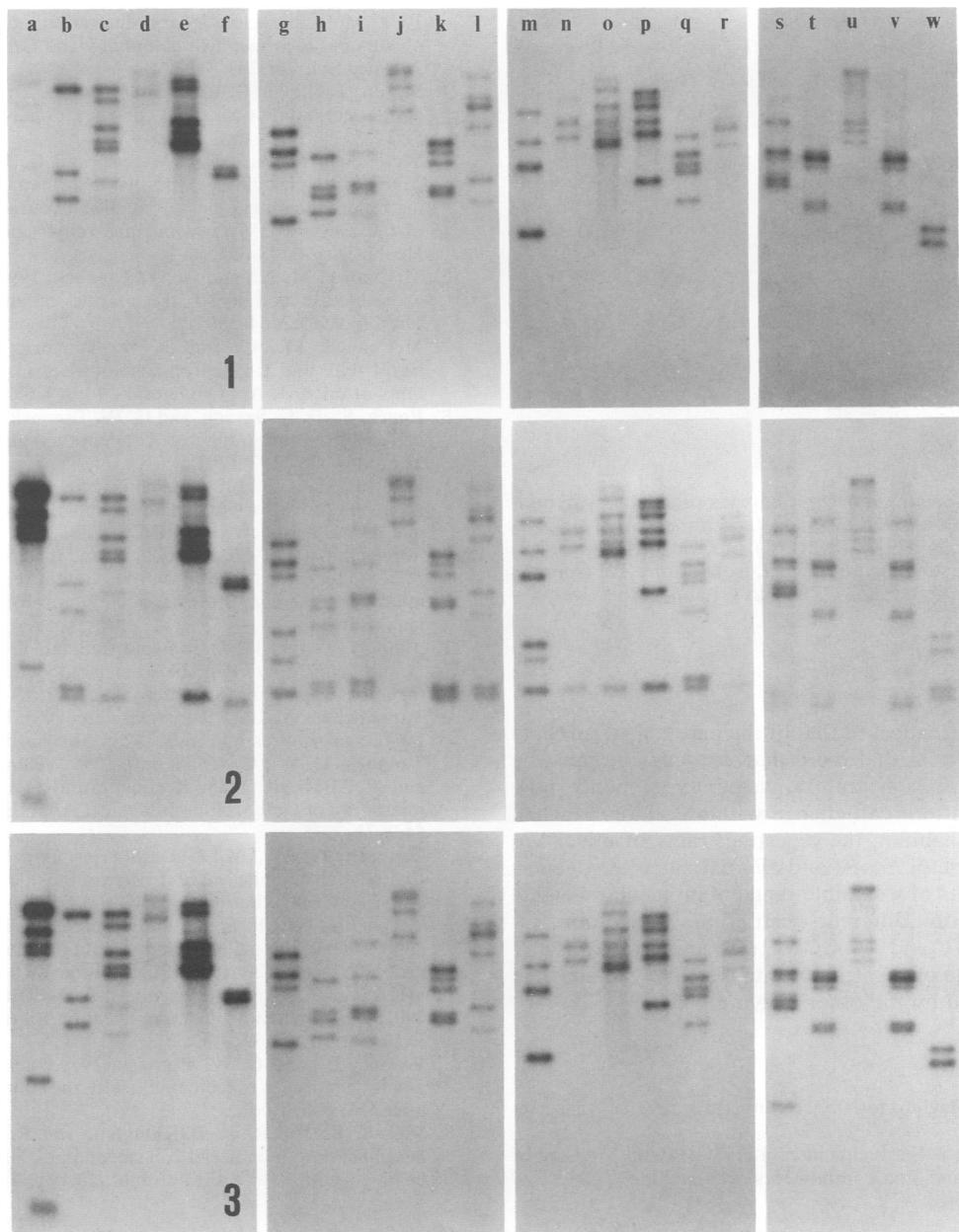


FIG. 2. Autoradiograms showing ribotypes produced by hybridization of three different probes to DNA from *E. coli* and *Lactobacillus* strains digested by *Hind*III. Row 1, *L. acidophilus* ATCC 4356 16S rDNA as probe; row 2, *E. coli* cDNA of 16S+23S rRNA as probe; row 3, *E. coli* 16S rDNA as probe. DNA in lanes a to w in all rows is the same as in Fig. 1.

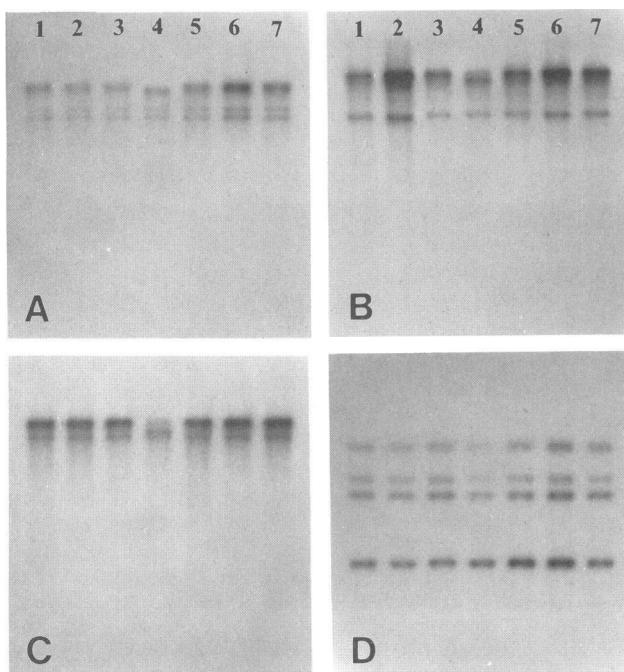


FIG. 3. Autoradiograms showing ribotypes produced by hybridization of *E. coli* cDNA (16S+23S) to *L. delbrueckii* GT21 DNA digested by *Bc*II (A), *Bg*/II (B), *Eco*RI (C), or *Hind*III (D). Lanes: 1, *L. delbrueckii* GT21 stock culture; 2 to 7, isolates of GT21 from the digestive tracts of mice (strains GT21/1 to GT21/6, respectively).

parameters were identical to that of the parental strain (data not shown). It is clear that more than one enzyme must be used to characterize each strain, since our experiments show that a ribotype pattern can change within 22 months when lactobacilli are inhabiting the digestive tracts of mice. We recommend the use of *Eco*RI and *Hind*III since they generate DNA fragments of a suitable range of molecular weights and have markedly different restriction recognition sequences (G/AATT and A/AGCTT, respectively). Thus any base substitution in one restriction site is unlikely to alter the ribotype generated by the other enzyme.

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Safety Evaluation of *Bifidobacterium breve* IDCC4401 Isolated from Infant Feces for Use as a Commercial Probiotic

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Previously, our research group isolated *Bifidobacterium breve* IDCC4401 from infant feces as a potential probiotic. For this study, we evaluated the safety of *B. breve* IDCC4401 using genomic and phenotypic analyses. Whole genome sequencing was performed to identify genomic characteristics and investigate the potential presence of genes encoding virulence, antibiotic resistance, and mobile genetic elements. Phenotypic analyses including antibiotic susceptibility, enzyme activity, production of biogenic amines (BAs), and proportion of D-/L-lactate were evaluated using E-test, API ZYM test, high-performance liquid chromatography (HPLC), and D-/L-lactic acid assay respectively. The genome of *B. breve* IDCC4401 consists of 2,426,499 bp with a GC content of 58.70% and 2,016 coding regions. Confirmation of the genome as *B. breve* was provided by its 98.93% similarity with *B. breve* DSM20213. Furthermore, *B. breve* IDCC4401 genes encoding virulence and antibiotic resistance were not identified. Although *B. breve* IDCC4401 showed antibiotic resistance against vancomycin, we confirmed that this was an intrinsic feature since the antibiotic resistance gene was not present. *B. breve* IDCC4401 showed leucine arylamidase, cystine arylamidase, α -galactosidase, β -galactosidase, and α -glucosidase activities, whereas it did not show production of harmful enzymes such as β -glucosidase and β -glucuronidase. In addition, *B. breve* IDCC4401 did not produce any tyramine, histamine, putrescine, cadaverine, or 2-phenethylamine, which are frequently detected BAs during fermentation. *B. breve* IDCC4401 produced 95.08% of L-lactate and 4.92% of D-lactate. Therefore, our findings demonstrate the safety of *B. breve* IDCC 4401 as a potential probiotic for use in the food industry.

Keywords: Safety evaluation, *Bifidobacterium breve*, probiotics

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Introduction

The United Nations and World Health Organization (WHO) [1] define probiotics as “live microorganisms which confer health benefits on the host when administered in adequate amounts”. Probiotics provide their major health benefits by inhibiting the growth of pathogens in the gastrointestinal tract, reducing the risk of colon cancer and bowel disease, controlling serum cholesterol levels, facilitating digestion, and improving nutrient absorption [2-5]. In addition, they contribute to a balanced gut microbial community and strengthen the immune system. Due to these health benefits and advantages, customer interest in probiotic foods has been increasing. Probiotics have been applied in a wide range of industries such as food, alcoholic beverage, periodontal disease treatment, animal feed and cosmetics [4, 6]. As a result, the global probiotic market size was estimated at approximately 48 billion USD in 2018 and is predicted to reach 77.09 billion USD with a compound annual growth rate of 6.9% by 2025 [7].

Most probiotics are gram-positive, catalase-negative, and non-pathogenic bacteria. There are several bacteria that are used as probiotics such as *Lactobacillus* spp., *Bifidobacterium* spp., *Propionibacterium* spp., *Peptostreptococcus productus*, *Bacillus* spp., *Lactococcus* spp., *Enterococcus faecium*, *Pediococcus* spp., and *Streptococcus* spp.. Of these, *Lactobacillus* spp. and *Bifidobacterium* spp. were the most commonly used as probiotics in the food and pharmaceutical industries [4, 8]. *Bifidobacterium* spp. were first isolated from the feces of breast-fed infants in 1899 [9] and are among the most dominant probiotics during the neonatal period, especially in breast-fed infants [10]. Previous studies have suggested that *Bifidobacterium* spp. are predominantly found in the feces of healthy breast-fed infants contributing to decreased infant diarrhea [11] as well as providing various health benefits as a

probiotic [3, 12]. In particular, *Bifidobacterium* spp. play an important role in balancing the gut microbiota such that the food and pharmaceutical industries use *Bifidobacterium* spp. as a starter culture in their products [13]. Therefore, searching new lactic acid bacteria for commercial application is required.

Recently, our research group isolated *B. breve* IDCC4401 from infant feces in Korea. Although *Lactobacillus* and *Lactococcus* are considered as “generally recognized as safe (GRAS),” *Bifidobacterium* spp. require safety evaluation [14]. For example, some probiotics possess genes encoding virulence, antibiotic resistance, and mobile genetic elements, and have deleterious metabolic activities [production of D-lactate, enzyme activity, and biogenic amines (BAs)] [4, 10, 15–17]. Thus, an FAO/WHO Expert Consultation has recognized and emphasized the necessity for systematic evaluation guidelines for probiotics prior to their commercialization [18]. Following these guidelines for probiotics in food, the safety of isolated novel strain of *B. breve* IDCC4401 was evaluated through genomic analysis to determine the presence of virulence genes, antibiotic resistance genes, and mobile genetic elements. In addition, *B. breve* IDCC4401 was examined for antibiotic susceptibility, enzyme activity, production of BAs, and the proportion of D-/L-lactate formed during incubation.

Materials and Methods

Bacterial Culture and Growth Conditions

B. breve IDCC4401 obtained from Ildong BioScience Co. (Korea) and grown in 15 ml of MRS broth (Difco Laboratories Inc., USA) at 37°C for 24 h with 0.5% CO₂ in a static incubator. After centrifugation at 6,000 ×g for 15 min, the pellet was resuspended in PBS (pH 7.4; Life Technologies Ltd., UK) and its concentration was adjusted to 10⁸ CFU/ml and 10⁹ CFU/ml by measuring the optical density at 640 nm.

Whole Genome Sequencing of *B. breve* IDCC4401

Genomic DNA of *B. breve* IDCC4401 was extracted using a Maxwell 16 LEV Blood DNA Kit and a Maxwell 16 Buccal Swab LEV DNA Purification Kit (Promega Co., USA) according to the manufacturer’s instructions. Genome sequencing was performed by Macrogen Inc. (Korea) using a PacBio RS II instrument (Pacific Biosciences of California Inc., USA) with an Illumina platform (Illumina Inc., USA). Raw data were assembled using the Hierarchical Genome Assembly Process and the assembled gene was predicted and annotated using Prokka v1.13. For additional annotation, predicted protein sets were then analyzed using InterProScan v5.30-69.0 and psiblast v2.4.0 with EggNOG DB v4.5. Average nucleotide identity (ANI) was calculated using average nucleotide identity.py script of PYANI v0.2.10 with ANIm option. To confirm the presence of antibiotic resistance genes and virulence factors, all identified coding sequences (CDS) were compared against the Comprehensive Antibiotic Resistance Database (CARD) [19] and Virulence Factor Database (VFDB) [20], respectively. Prophage regions were identified using the PHASTER web-based program [21]. Transposases were annotated using BLASTP and transposases and conjugal transfer proteins were retrieved from the NCBI GenBank. Circular maps depicting each contig were generated using Circos v0.69.3. The complete genome sequence of *B. breve* IDCC4401 was made available in the GenBank database under nucleotide sequence accession number KP325411.

Antibiotic Susceptibility of *B. breve* IDCC4401

The antibiotic susceptibility of *B. breve* IDCC4401 was determined by the E-test method against 9 antibiotics according to the European Food Safety Authority (EFSA) guidelines [17]. An overnight culture of bacteria (10⁸ CFU/ml) was swabbed onto 15-cm diameter MRS agar plates with a sterilized cotton swab prior to placing an E-test strip (Liofilchem Inc., Italy) on the surface of the plate. After incubation at 37°C for 18 h, the relevant inhibition ellipse intersected the strip and the minimum inhibitory concentration was determined at complete inhibition. Finally, antibiotic susceptibility of *B. breve* IDCC4401 was determined following the guidelines of the EFSA [17].

Enzyme Activities of *B. breve* IDCC4401

The enzyme activities of *B. breve* IDCC4401 were determined using the API ZYM Kit (Biomerieux Inc., France) according to the manufacturer’s instructions. The overnight culture of *B. breve* IDCC4401 (10⁹ CFU/ml) was added into a cupule containing different substrate solutions and incubated at 37°C for 4 h. One drop of ZYM A and ZYM B reagents was added sequentially prior to incubation for 5 min at RT. Color change of the mixture was graded from zero (no activity) to five by comparing color intensity with the color chart provided by the manufacturer. Positive enzyme activities were determined to be above three intensity levels of color change.

BA Production of *B. breve* IDCC4401

Production of BAs by *B. breve* IDCC4401 was investigated following the method described in a previous study [22] with minor modifications. The overnight cultured *B. breve* IDCC4401 was centrifuged at 2,300 ×g for 5 min at 4°C. An aliquot of 0.75 ml of supernatant was mixed with the same volume of 0.1 M HCl and filtered through a 0.45-μm membrane to extract BAs. For derivatization of the BAs, 1 ml of filtered BAs was incubated at 70°C for 10 min prior to addition of 200 μl of saturated NaHCO₃, 20 μl of 2 M NaOH, and 0.5 ml of dansyl chloride solution (10 mg/ml of acetone). The derivatized BAs were mixed with 200 μl of proline (100 mg/ml of H₂O) and incubated for 15 min at RT in the dark. The derivatized BAs were then separated and quantified using HPLC (LC-NETII/ADC, JASCO Inc., Japan) with an Athena C18 column (4.6 mm × 250 mm, ANPEL Laboratory Technologies Inc., China). Aqueous acetonitrile solution (Sigma-Aldrich Co., USA) was used as a mobile phase and the flow rate was adjusted to 0.8 ml/min. Finally, a peak was detected at 254 nm using a UV detector (UV-2075 Plus, JASCO Inc.,

Japan). The detected BAs were quantified from calibration curves of BAs including tyramine, histamine, putrescine, 2-phenethylamine, and cadaverine (Sigma-Aldrich Co.).

Proportion of D-/L-Lactate of *B. breve* IDCC4401

An overnight culture of *B. breve* IDCC4401 was centrifuged at 2,300 × g for 30 min at 4°C and the supernatant was collected. Following supernatant filtration using a 0.2-μm pore size membrane, the filtrate was mixed with the agents in a D-/L-Lactic Acid (D-/L-Lactate) (Rapid) Assay Kit (Megazyme, Ireland). Absorbances of the mixture were measured at 340 nm and the concentration of D-/L-lactate was calculated according to the manufacturer's protocol.

Results and Discussion

Whole Genome Sequencing of *B. breve* IDCC4401

Whole genome sequencing of *B. breve* IDCC4401 was performed for the identification and confirmation of genes encoding antibiotic resistance, virulence, and mobile genetic elements to ensure safety. The assembled genome consisted of 2,426,499 bp with a GC content of 58.70% (Fig. 1). The strain was confirmed as *B. breve* with a similarity of 98.93% with *B. breve* DSM20213 based on ANI analysis. Of a total of 2,016 CDSs of *B. breve* IDCC4401, 1,583 CDSs were annotated as functional genes involved in translation, ribosomal structure, biogenesis, RNA processing, modification, transcription, replication, recombination, repair, cell cycle control, cell division, chromosome partitioning, defense mechanisms, signal transduction mechanisms, and 433 unknown genes (Table 1). Based on CARD and VFDB, genes associated with virulence and antibiotic resistance were not found in *B. breve* IDCC4401, respectively. Although 33 transposases, as mobile genetic elements, were identified, these mobile elements were not involved in the acquisition and transfer of antibiotic resistance genes due to the absence of virulence and antibiotic resistance genes in *B. breve* IDCC4401. Therefore, this result confirmed the safety of *B. breve* IDCC4401 for use as a probiotic based on our thorough genome analysis.

Antibiotic Susceptibility (MICs) of *B. breve* IDCC4401

To ensure safety, the phenotypic antibiotic susceptibility of *B. breve* IDCC4401 was investigated against 9 antibiotics including ampicillin (one representative of a β-lactam antibiotic), gentamicin, streptomycin, erythromycin, clindamycin, tetracycline, chloramphenicol, vancomycin, and kanamycin, using the E-test method [18]. Determination of resistance and susceptibility against each antibiotic followed the EFSA guidelines. As shown in Table 2, *B. breve* IDCC4401 was susceptible to ampicillin, gentamicin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol but resistant to vancomycin. Susceptibility to kanamycin could not be determined because EFSA did not provide a definite cut-off value. However, the MIC value (256 µg/ml) against kanamycin was 2 times lower than that of *B. longum* BORI (512 µg/ml), and 4 times lower than those of *B. longum* BB536, *B. breve* M-16, *B. bifidum* BGN4, and *B. lactis* BB-12 (1,024 µg/ml) that were considered as GRAS [16].

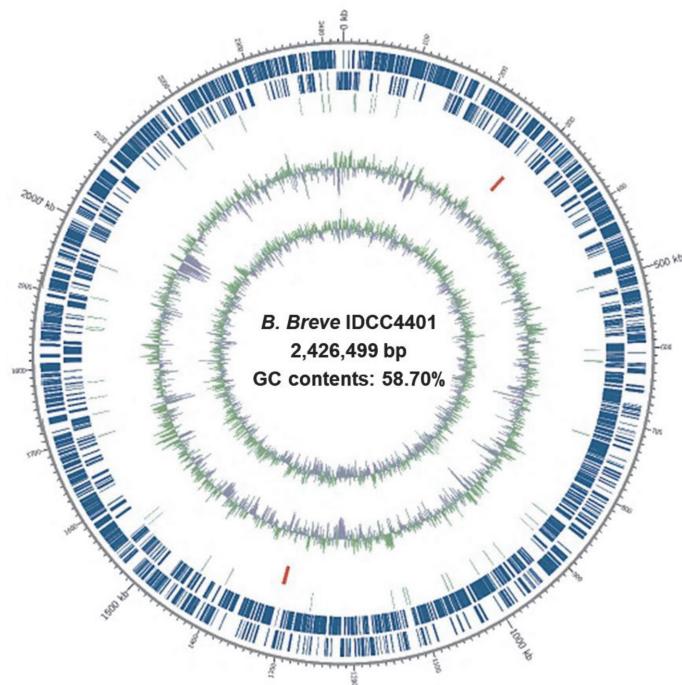


Fig. 1. Genomic map of *B. breve* IDCC4401. Marked genome characteristics are shown from outside to the center; CDS on forward strand, CDS on reverse strand, tRNA, rRNA, GC content and GC skew.

Table 1. Annotated functional genes in *B. breve* IDCC4401.

Function	Number of CDS	Ratio (%) of CDS
Translation, ribosomal structure, and biogenesis	134	6.6468
RNA processing and modification	1	0.0496
Transcription	125	6.2004
Replication, recombination, and repair	191	9.4742
Cell cycle control, cell division, chromosome partitioning	19	0.9425
Defense mechanisms	52	2.5794
Signal transduction mechanisms	53	2.6290
Cell wall/membrane/envelope biogenesis	90	4.4643
Intracellular trafficking, secretion, and vesicular transport	14	0.6944
Posttranslational modification, protein turnover, chaperones	51	2.5298
Energy production and conversion	47	2.3313
Carbohydrate transport and metabolism	231	11.4583
Amino acid transport and metabolism	166	8.2341
Nucleotide transport and metabolism	62	3.0754
Coenzyme transport and metabolism	34	1.6865
Lipid transport and metabolism	28	1.3889
Inorganic ion transport and metabolism	95	4.7123
Secondary metabolites biosynthesis, transport and catabolism	9	0.4464
General function prediction only	181	8.9782
Unknown function	433	21.4782

Table 2. Minimal inhibitory concentration (MIC) and antibiotic susceptibility of *B. breve* IDCC4401.

	Cut-off value ($\mu\text{g/ml}$)	MIC ($\mu\text{g/ml}$)	Assessment
Ampicillin	2	0.25–0.5	S ¹
Vancomycin	2	>512	R ²
Gentamicin	64	32	S
Kanamycin	– ³	256	–
Streptomycin	128	64–128	S
Erythromycin	1	0.125–0.25	S
Clindamycin	1	0.25	S
Tetracycline	8	2	S
Chloramphenicol	4	4	S

¹S, Susceptible²R, Resistant³–, Cut-off value is not established in EFSA guidelines.

Charteris *et al.* (1999) investigated the antibiotic susceptibilities of two *B. breve* strains (15698 and 15701) isolated from human gastrointestinal tract and found resistances against five β -lactam antibiotics (penicillin G, amoxycillin, cepharadine, ceftizoxime and cefotaxime) among 44 antibiotics considered as “last resort antibiotics” [23]. In the meantime, *B. breve* IDCC4401 exhibited susceptibility to ampicillin. In addition, two other *B. breve* strains (IF2-173 and IF2-174) also isolated from breast-fed infants were susceptible to ampicillin, streptomycin, and chloramphenicol whereas they were resistant to tetracycline, erythromycin, and vancomycin [10]. When compared with previous study [10], *B. breve* IDCC4401 showed narrow antibiotic resistance only to vancomycin. Although *B. breve* IDCC4401 showed antibiotic resistance against vancomycin, this result could be related to an intrinsic feature of *B. breve* IDCC4401 as vancomycin resistance is a general feature of most *Bifidobacterium* spp. as shown in a study by Charteris *et al.* [23]. Vancomycin resistance is thought to be a result of the presence of D-alanine residues in the cell wall preventing vancomycin binding [24, 25].

Enzyme Activity of *B. breve* IDCC4401

The enzyme activity of *B. breve* IDCC4401 was investigated against 19 different enzymes using the API ZYM assay, which involved degradation of peptides, phosphomonoesters, lipids, mucopolysaccharides, polysaccharides, chitin, cellulose, starch, and galactan (Table 3) [26]. For example, leucine and cysteine arylamidases are involved in the degradation of peptides whereas α -galactosidase, β -galactosidase, and α -glucosidase participate in the degradation of carbohydrates. *B. breve* IDCC4401 exhibited leucine arylamidase, cysteine arylamidase, α -galactosidase, β -galactosidase, and α -glucosidase activities among the 19 different enzymes.

Desjardins *et al.*'s study demonstrated that four *B. breve* strains (ATCC 15698, ATCC 15699, ATCC 15700, and ATCC 15701) obtained from the American Type of Culture Collection (ATCC) exhibited the activities of esterase lipase, leucine aminopeptidase, acid phosphatase, phosphoamidase, α -galactosidase, β -galactosidase, α -glucosidase, and β -glucosidase [27]. In addition, *B. breve* ATCC 15699 showed β -glucuronidase activity. The enzyme activities of α -galactosidase, β -galactosidase and α -glucosidase were found to be consistent with those of a previous study [27]. Chevalier *et al.* (1990) demonstrated that the presence of *Bifidobacterium* spp. in the feces of the healthy

Table 3. Enzyme activities of *B. breve* IDCC4401 determined by API ZYM test.

Enzyme	Activity*
Alkaline phosphatase	—
Esterase	—
Esterase Lipase	—
Lipase	—
Acid phosphatase	—
Naphthol-AS-BI-phosphohydrolase	—
Leucine arylamidase	+
Valine arylamidase	—
Cystine arylamidase	+
Trypsin	—
α-Chymotrypsin	—
α-Galactosidase	+
β-Galactosidase	+
β-Glucuronidase	—
α-Glucosidase	+
β-Glucosidase	—
N-acetyl-β-glucosaminidase	—
α-Mannosidase	—
α-Fucosidase	—

*+, enzyme activity; —, no enzyme activity

infants was accompanied by α-galactosidase and α-glucosidase, which are characteristics of *Bifidobacterium* spp. [28]. Further, evidence proved that α-galactosidase and β-galactosidase enzyme activities were highly produced in *B. longum* RD 47, suggesting that these enzymes are involved in hydrolyzing galactosides such as lactose [29]. These findings are consistent with our results obtained from this study. Although probiotics can improve digestion and probiotic enzymes can act as natural substances for digestion of food in the human body, some enzyme activities may produce compounds that are to the host [30]. Cole and Fuller (1986) reported that β-glucosidase may produce aglycones which are linked to the development of colorectal cancer [31]. Moreover, β-glucuronidase might be linked to carcinogenic compounds for colorectal cancer [32]. Since *B. breve* IDCC4401 did not show the activity of β-glucosidase and β-glucuronidase, it appears to be safe and suitable for use as a probiotic.

BA Production of *B. breve* IDCC4401

Lactic acid bacteria (LAB) including *Bifidobacterium* spp. and *Lactobacillus* spp. can produce BAs during the fermentation process [33]. Previous studies [4, 34] reported that tyramine, histamine, putrescine, cadaverine, 2-phenethylamine, and spermidine can be frequently detected in fermented foods. The problem is that these BAs, at high concentration, can cause toxicological effects to humans with certain symptoms such as respiratory distress, heart palpitation, hypertension or hypotension, headaches, and allergic disorders [33]. Verifying the formation of BAs by *B. breve* IDCC4401 is required to ensure the safety of *B. breve* IDCC4401. As shown in Table 4, *B. breve* IDCC4401 did not produce any of the five representative BAs, indicating that it did not produce any harmful BAs.

According to Lorencova *et al.*'s study [35], five *Bifidobacterium* strains (*Bifidobacterium* spp., *B. adolescentis*, *B. lactic*, *B. bifidum*, and *B. longum*) produced tyramine, cadaverine, putrescine, and spermidine among the eight BAs including the five we tested and three others (tryptamine, speramine, and spermidine). However, Kim *et al.*'s study [16] exhibited putrescine formation by *B. bifidum* BGN4 and *B. longum* BORI, but not cadaverine, histamine, or tyramine formation. Contradictory to previous studies, Ku *et al.* [4] demonstrated that *B. lactic* AD011 did not form any BAs when tested for cadaverine, histamine, putrescine, and tyramine formation, in accordance with our results. Thus, the absence of BAs suggested the potential of *B. breve* IDCC4401 as a commercial probiotic.

Proportion of D-/L-Lactate of *B. breve* IDCC4401

Lactate produced in either D-form or L-form isomers during fermentation by LAB and exhibits distinct biological effects in human [36]. However, *Lactobacillus* and *Bifidobacterium* have been known as D-lactate producers [37, 38]. Unlike L-lactate, excess D-lactate produced by LAB cause short bowel syndrome, chronic fatigue and metabolic disorders, especially when jejunoileal bypass surgery is performed [39]. Due to these

Table 4. Biogenic amine production of *B. breve* IDCC4401.

Biogenic amine (μg/ml)				
Tyramine	Histamine	Putrescine	Cadaverine	2-Phenethylamine
ND.	ND.	ND.	ND.	ND.

ND: not detected.

Table 5. L-/D-lactate production of *B. breve* IDCC4401.

L-lactate (g/l)	D-lactate (g/l)	L-form (%)	D-form (%)
21.26	0.93	95.08	4.92

The data represent the mean \pm SD ($n = 3$).

toxicological effects, the proportion of D-/L-lactate of *B. breve* IDCC4401 needs to be clarified for its safety evaluation. As shown in Table 5, *B. breve* IDCC4401 produced 95.08% (21.26 g/l) of L-lactate and 4.92% (0.93 g/l) of D-lactate.

Since the guidelines provided by FAO/WHO did not provide any clear criterion for the ratio of L-lactate and D-lactate, these results were compared with other studies recognized as GRAS. According to Munoz *et al.*'s study [40], *B. longum* CECT 7210 produced 2.22% (0.06 g/l) of D-lactate, which was lower than the L-lactate (97.78%, 2.64 g/l). Although the amount and ratio of D-lactate produced by *B. breve* IDCC4401 was higher than that of *B. longum* CECT 7210, it was lower than that of L-lactate and this pattern was similar to a previous study. In the case of *B. lactis* BB-12⁺ which was certified as GRAS by the FDA, the ratio of L-lactate was more than 95% [41]. In this aspect, it can be assumed that *B. breve* IDCC4401 poses no safety concern over this property.

In conclusion, since the safety of *B. breve* IDCC4401 isolated from infant feces has not yet been investigated for its commercial usage as a probiotic, we investigated the presence of genes encoding virulence, antibiotic resistance, mobile genetic elements, and deleterious metabolic activities in this strain to ascertain its safety as a probiotic. Although *B. breve* IDCC4401 was evaluated for safety in this study, further studies are required on functional characteristics such as resistance to gastric acid and bile salts, adherence ability (aggregation properties and cell hydrophobicity), and antimicrobial activity to ensure its safety as a commercial probiotic in the food industry.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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Selective enumeration and identification of mixed cultures of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *Bifidobacterium lactis* in fermented milk

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Abstract

This study describes selective plating methodologies for enumeration of mixed cultures of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *Bifidobacterium lactis* in fermented milk based on selective antibiotic-free media. Enumeration of *S. thermophilus* was performed using M17-lactose. MRS-fructose was suitable for enumeration of *L. bulgaricus* and MRS-maltose for differentiation between *L. acidophilus* and *L. paracasei*. The selective enumeration of *B. lactis* was obtained using MRS-raffinose containing 0.05% LiCl. The bacterial counts obtained using selective methods were equivalent to those under optimum culture conditions at a probability level of 95%. Performance of the methods was verified in fermented milk products where identification of the enumerated species was confirmed by species-specific polymerase chain reaction. This study shows that combination of species-specific polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) analysis has great detection and identification potential for verification of accurate species labelling in fermented milk without prior isolation of the bacteria.

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Keywords: Selective methods; Lactobacilli; Bifidobacteria; Fermented milk; PCR-DGGE

1. Introduction

The CODEX standard for fermented milk products (CODEX STAN 243-2003) establishes them as the products obtained by fermentation of milk by the action of suitable starter microorganisms that should be viable, active and abundant in the product to the date of minimum durability (Codex Alimentarius Commission, 2003). The name yoghurt should be used when the milk is only fermented by *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Regarding viability, the norm specifies that the sum of microorganisms constituting the starter culture should be at least 10^7 cfu g⁻¹, and that minimum counts of other labelled microorganisms should

be 10^6 cfu g⁻¹. Therefore, microbial viability and authenticity are prominent criteria to be analytically verified for the compliance of fermented milk with the required product specifications. Likewise, probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit in the host (FAO/WHO, 2002). However, the minimum amount of probiotics needed to obtain a clinical effect has not been established. As more information on probiotics is available, it seems likely that numbers will vary as a function of the strain and the health effect desired (Roy, 2005).

Fermented milk products are the most popular means of delivering probiotic bacteria in food. Among them, strains of *L. acidophilus*, *L. casei* complex and *Bifidobacterium lactis* predominate in commercial probiotic products (Fasoli et al., 2003; Gueimonde et al., 2004; Masco, Huys, De Brandt, Temmerman, & Swings, 2005; Yeung, Sanders,

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Kitts, Cano, & Tong, 2002). The presence of multiple and closely related species in these products makes the differential enumeration of probiotic and yoghurt starter bacteria difficult due to similarity in growth requirements and overlapping biochemical profiles of the species. Numerous media have been proposed for selective and differential enumeration of lactobacilli and bifidobacteria in mixed bacterial populations, and some have been the subject of specific reviews (Charteris, Kelly, Morelli, & Collins, 1997; Shah, 2000; Roy, 2001; Coeuret, Dubernet, Bernardeau, Gueguen, & Vernoux, 2003) and of comparative performance analyses (Payne, Morris, & Beers, 1999; Talwalkar & Kailasapathy, 2004; Masco et al., 2005; Van de Castele et al., 2006). In order to cover a high spectrum of species, most media for selective enumeration of mixed cultures have complex compositions that include antibiotics as selective ingredients, which could impact on the response of not only the sensitive strains but also of target bacteria and result in inaccurate or irreproducible quantitative results. A comparison of methods described in literature (Talwalkar & Kailasapathy, 2004) concluded that no reliable techniques are yet developed to accurately enumerate *L. acidophilus*, *L. casei* and *Bifidobacterium* in different commercial yoghurts. Overall, it seems rational that the choice of selective methods should focus on the type of food and the species, even strains, to enumerate in each particular situation (Lourens-Hattingh & Viljoen, 2001; Sartory, 2005).

As previously stated, identification of species is another important issue to be verified for the compliance of fermented milk with the required product specifications in terms of accurate species labelling and, if appropriate, to support health claims that could be associated with added probiotics. Phenotypic methods alone are inadequate for identification of lactobacilli and bifidobacteria species (Dellaglio & Felis, 2005). To achieve a rapid and reliable identification of species, polymerase chain reaction (PCR)-based methods using species-specific primers targeting the 16S rRNA gene sequence diversity have become very popular (Coeuret et al., 2003). In addition, culture-independent methods for bacterial identification based on genetic analysis have become a valuable tool, since these techniques have the advantage to analyze the product as a whole. Separation of genus or species-specific PCR products by denaturing gradient gel electrophoresis (DGGE) has become the most commonly used technique among the culture-independent methods for detection and identification of lactobacilli and bifidobacteria from fermented products (Ercolini, 2004).

The aim of this study was to develop selective plating methodologies for enumeration and identification of mixed cultures of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis* in fermented milk products based on selective antibiotic-free media and different incubation conditions. To evaluate the performance of selective media for complete recovery of viable bacteria, methods were validated on the basis of

their precision, accuracy, reproducibility, selectivity and specificity characteristics, in relation to culture conditions, which were used as reference methods. Efficacy of the selective methods was verified by identification of the presumptive colonies using species-specific PCR. The study is also complemented with the application of a culture-independent procedure based on PCR-DGGE analysis to the rapid detection and identification of the mixed species in fermented milk products.

2. Material and methods

2.1. Microorganisms and culture conditions

Strains used in the assay were *S. thermophilus* STY-31, *L. delbrueckii* subsp. *bulgaricus* LBY-27, *L. acidophilus* LA-5, *L. paracasei* subsp. *paracasei* LC-01, and *B. lactis* BB-12. The strains were purified from a commercial symbiotic product (Simbiotic Drink; Priégola, Madrid, Spain). To allow the correct identification of strains, 16S rRNA gene nucleotide sequencing was carried out from pure cultures. The entire gene was amplified using the primers *SacI*-POmod and *SaII*-T7-PC5 (Table 1) and the PCR conditions described previously by Rodtong and Tannock (1993). Additional primers used to assist in sequencing were 16Smidfor and P3rev (Table 1). Sequencing of PCR fragments was carried out for both strands at the DNA Sequence Service of the Centro de Investigaciones Biológicas-CSIC (Madrid, Spain). *S. thermophilus* was grown in M-17 broth (Pronadisa, Madrid, Spain) containing 2% lactose. *Lactobacillus* subsp. and *B. lactis* were grown under anaerobic conditions (Gas-Pack, Anaerogen; Oxoid Ltd., Hampshire, England) in MRS broth (Pronadisa) supplemented with 0.05% L-cysteine hydrochloride, excepting *L. paracasei* subsp. *paracasei* that was grown aerobically in MRS broth. Incubations were carried out for 18–24 h at 37 °C and at 30 °C for *L. paracasei* subsp. *paracasei*.

2.2. Selective methods

Culture conditions described above were selected as reference methods. Media were supplemented with 1.5% bacteriological agar (Scharlab, Barcelona, Spain) and incubation extended to 48 h for *S. thermophilus* and 72 h for *B. lactis* and lactobacilli.

The selective conditions for the enumeration of *S. thermophilus* included inoculation of appropriate dilutions by the pour-plate technique into M-17 agar containing 1% lactose (M17-lactose) and incubation at 45 °C for 24 h. For enumeration of *L. delbrueckii* subsp. *bulgaricus*, appropriate dilutions were pour-plated into MRS fermentation broth (Pronadisa), which does not contain either glucose or meat extract (De Man, Rogosa, & Sharpe, 1960), enriched with 0.2% Tween 80 and supplemented with 1% fructose, 0.8% casein acid hydrolysate, 0.05% cysteine, and 1.5% agar (MRS-fructose). Plates were incubated in anaerobic

Table 1

Polymerase chain reaction primers used in this study for the identification of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis*

Species	Name	Sequence 5' → 3'	Product (bp)
<i>S. thermophilus</i>	Thermfor	ACGCTGAAGAGAGGGAGCTTG	157
	Thermrev	GCAATTGCCCTTCAAATA	
<i>L. bulgaricus</i>	Bulgfor	TCAAAGATTCCTTCGGGATG	232
	Bulgrev	TACGCATCATTGCCTGGTA	
<i>L. acidophilus</i>	Acidfor	AGCGAGCTGAACCAACAGAT	227
	Acidrev	AGGCCGTTACCCCTACCAACT	
<i>L. paracasei</i>	Casfor	GCACCGAGATTCAACATGGAA	142
	Casrev	GCCATCTTCAGCCAAGAAC	
<i>B. lactis</i>	Forlac	GCGCTGGGCTGCTCTGGAAGC	116
	Revlac	TGGCGACGAGCTCATGCACATACT	
All species	SacI-POmod	CCGAGCTCAACAGAGTTGATCCTGGCTCAG	792–825 ^a
	P3rev	GGACTACCAGGGTATCTAAT	
	16Smidfor	GGCCGTTACTGACGCTGAG	767–771 ^a
	SaII-T7-PCS	GGTCGACCGTTAACAGACTCACTATAGGGATACCTTGTACGACTT	

^aSize range of products obtained from the five species.

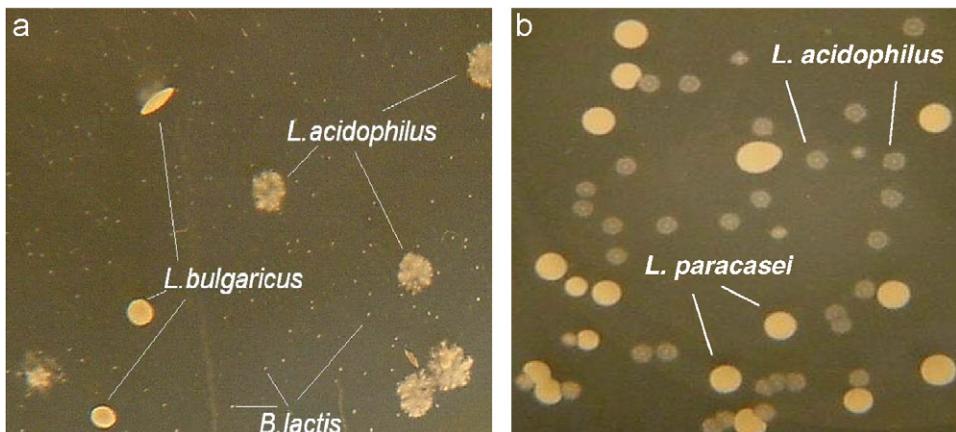


Fig. 1. Differentiation of *L. acidophilus* from *L. delbrueckii* subsp. *bulgaricus* and *B. lactis* on MRS-fructose agar (a) and from *L. paracasei* subsp. *paracasei* on MRS-maltose agar (b).

jars at 45 °C for 72 h and lenticular colonies with 1–2 mm diameter were enumerated as *L. delbrueckii* subsp. *bulgaricus*, whereas cottony–fluffy colonies of 2–3 mm diameter corresponded to *L. acidophilus* (Fig. 1a). Enumeration of *L. acidophilus* was performed by spreading out appropriate dilutions onto MRS fermentation broth enriched with 0.2% Tween 80 and supplemented with 1% maltose, 0.05% cysteine, and 1.5% agar (MRS-maltose). Plates were incubated in a 20% CO₂ atmosphere incubator at 37 °C for 72 h. Flat, rough colonies with irregular edges and 1–2 mm diameter corresponded to *L. acidophilus*, whereas *L. paracasei* subsp. *paracasei* developed as white, smooth and circular colonies of 2–3 mm diameter (Fig. 1b). The method was also selected for the enumeration of *L. paracasei* subsp. *paracasei*. Enumeration of *B. lactis* was carried out by pour-plating appropriate dilutions into MRS fermentation broth supplemented with 1% raffinose, 0.05% LiCl, 0.05% cysteine, and 1.5% agar (MRS-raffinose). Plates were incubated in anaerobic jars at 45 °C for 72 h.

2.3. Efficiency tests

To evaluate performance of the selective methods to enumerate lactic acid bacteria (LAB) and *B. lactis*, recommendations of the ISO/TR 13843 (ISO, 2000) and ISO/IEC 17025 (ISO, 2005) standards on validation of microbiological methods were followed. Parameters evaluated were precision, accuracy, reproducibility, selectivity and specificity. The precision and accuracy of the methods were determined by the comparison between the bacterial counts obtained with selective and reference methods. Overnight pure cultures were diluted and inoculated into both selective and reference media. After logarithmic transformation of the results to normalize the distribution, counts obtained in both media were compared using paired Student's *t*-test to obtain $t_{\text{exp}} = d_m/(s_d/n^{1/2})$, where d_m is the mean of differences (d) between counts on selective and reference methods, s_d is the standard deviation of d , and n the number of samples. Other parameters calculated were relative recovery = 10^{-d_m} and relative standard deviation

of differences = $1 - 10^{-s_d}$. Reproducibility of the methods was tested by the analysis of identical samples by two different operators and using different equipments. At the same time, the matrix effect for bacterial enumeration in fermented milk was evaluated. Samples consisted of 10% reconstituted skim milk powder (Scharlab) acidified to pH 4.6 with 5 M lactic acid and inoculated with each species at both, high levels (10^7 cfu mL^{-1}) and low levels (10^5 cfu mL^{-1}). Pairs of counts were compared and the relative standard deviation of reproducibility was calculated as $1 - 10^{-s_{dr}}$, where s_{dr} is the standard deviation of differences between counts from the two operators. For selectivity and specificity analysis, cultures from all five bacterial species were mixed at the level of 10^7 cfu mL^{-1} for each strain and appropriate dilutions inoculated into the selective media. Results were expressed as the percentage of the presumptive target counts in relation to theoretical counts. In addition, 10% reconstituted skim milk, pH 4.6, was inoculated with cultures of each target strain at low level (10^5 cfu mL^{-1}) and mixed with the other four strains at high level (10^7 cfu mL^{-1}). Appropriate dilutions were plated and analysed for the presence of presumptive false positive and negative colonies. All analyses were performed at least in triplicate and differences were compared at a significance level of 0.05 by a Student's *t*-test using Excel software (Microsoft, Redmond, WA, USA).

2.4. Analysis of fermented milk products

2.4.1. Enumeration of bacterial viable counts

The fermented milk Simbiotic Drink containing the yoghurt and probiotic strains and the product with only the yoghurt bacteria, both from Priégola, were analysed through their shelf life (28 d) for performance of the selective methods. Viable counts were determined in samples (1 mL) by using serial decimal dilutions prepared in Ringer's solution (Scharlab) supplemented with 0.05% cysteine. Appropriate dilutions were plated in duplicate and analysed using the selective methods described above.

2.4.2. Identification of presumptive target colonies

Presumptive positive colonies (10%) grown with selective methods in the highest dilution plate were checked by species-specific PCR to verify the efficacy of the media for specific enumeration. Species-specific primers were designed within variable regions in the 16S rRNA encoding genes of *S. thermophilus*, *L. paracasei* subsp. *paracasei*, *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus* using the Lasergene PrimerSelect module of the Lasergene software package (DNAStar Inc., Madison, WI, USA). Species-specific primers for identification of *B. lactis* were designed on the basis of the transaldolase gene variable regions (Requena et al., 2002). Primer pairs (Table 1) were selected upon confirmation that only targeted species would give a PCR product. Colonies were picked up using sterile toothpicks, suspended in 20 µL milliQ water, boiled at 100 °C for 5 min and frozen at –20 °C. Diagnostic PCR

reactions were carried out with 2 µL of thawed cell suspensions and the primers described in Table 1. The amplification programme was as follows: 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 60 °C for 20 s and 72 °C for 20 s, and a final extension time of 72 °C for 5 min. The products (5 µL) were separated on a 2% agarose gel and analysed for the yield of amplicons with the expected sizes (Table 1).

2.4.3. PCR-DGGE analysis

In order to obtain bacterial DNA from fermented milk, the samples (3 mL) were neutralized to pH 6.5 with 1 M NaOH and cleared by adding 10 mL of 0.2% EDTA, pH 12, to cause casein micelle dispersion. The bacterial cells were collected by centrifugation at $10,000 \times g$ for 15 min and mixed (1:1) with glass beads (diameter, 150–212 µm; Sigma Chemical Co., St. Louis, MO, USA) for mechanical disruption by vortexing the ice-cooled suspensions four times over 4 min. Cell debris and glass beads were collected by centrifugation ($12,000 \times g$ for 5 min) and genomic DNA was obtained from the cell free extract as described by Meile, Rohr, Geissman, Herensperger, and Teuber (2001). The DNA was used as template (500 ng) for PCR amplification using the conditions described above. A 40-bp GC clamp (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3') was attached to either the forward or reverse primer (Table 1) to obtain PCR products suitable for separation by DGGE. Thus, the following species-specific primers were employed: Thermfor-GC and Thermrev for the identification of *S. thermophilus*, Bulgfor and Bulgrev-GC for *L. delbrueckii* subsp. *bulgaricus*, Acidfor-GC and Acidrev for *L. acidophilus*, Casfor-GC and Casrev for *L. paracasei* subsp. *paracasei*, and Forlac and Revlac-GC for *B. lactis*. An identification ladder containing equal amounts of PCR products from pure cultures was prepared.

DGGE was performed with a DCode system (Bio-Rad Laboratories, Hercules, CA, USA), using a 9% polyacrylamide gel with a 40–60% gradient of 7 M urea and 40% formamide that increased in the electrophoresis running direction. Electrophoresis was carried out in 20 mM Tris, 10 mM acetic acid and 0.5 mM EDTA ($0.5 \times$ TAE) buffer at 130 V and 60 °C for 4.5 h. Gels were stained with AgNO₃ as described by Sanguinetti, Dias-Neto, and Simpson (1994).

3. Results and discussion

3.1. Comparison of bacterial counts using selective and reference methods

This study has focused on the development of selective methods suited to recover the maximum population of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis* in fermented milk as compared with that of supporting optimal growth (reference methods) to avoid underestimation of the bacterial counts. Therefore, formulation of the methods was based on antibiotic-free media, carbohydrate

fermentation patterns and different incubation conditions. Incubation in aerobiosis at 45 °C during 24 h in M17-lactose agar was found suitable for selective enumeration of *S. thermophilus*, since it prevented the growth of *L. paracasei* subsp. *paracasei* found at 37 °C and that of *L. delbrueckii* subsp. *bulgaricus* and *B. lactis* that developed under anaerobic conditions. Extension of the incubation period for 48 h allowed the appearance of pinpoint colonies of *L. acidophilus*. Therefore, these incubation conditions provided selective characteristics for *S. thermophilus* enumeration, although the medium does not inhibit the growth of the other bacteria.

The finding of a non-antibiotic medium for selective enumeration of *L. delbrueckii* subsp. *bulgaricus* could not be based on the acidified MRS medium recommended by ISO/FDIS 7889 IDF 117 standard on enumeration of yoghurt characteristic microorganisms (ISO, 2002), since it also allowed growth of *L. acidophilus*, *L. paracasei* subsp. *paracasei*, and *B. lactis*. Increasing the incubation at 45 °C and replacement of glucose by fructose were conditions selective against *L. paracasei* subsp. *paracasei* and *B. lactis*, respectively. The method was differential against *L. acidophilus* when the medium was enriched with 0.2% Tween 80, showing a clear morphological differentiation between lenticular colonies corresponding to *L. delbrueckii* subsp. *bulgaricus* and cottony-fluffy colonies of *L. acidophilus* (Fig. 1a). The role of the compound to cause such peculiar colony morphology in *L. acidophilus* was not elucidated. Selective enumeration of *L. acidophilus* against *S. thermophilus* and *B. lactis* in fermented milk using MRS-maltose and incubation in a 20% CO₂ atmosphere was shown in a previous report (Martín-Diana, Janer, Peláez, & Requena, 2003). In the present study, the method also demonstrated to be selective against *L. delbrueckii* subsp. *bulgaricus* and differential against *L. paracasei* subsp. *paracasei*, since a clear difference in colony morphology could be assessed in the plates (Fig. 1b). Counts of *L. acidophilus* were similar when the two methods, growth in MRS-maltose and MRS-fructose, were compared (results not shown). MRS-maltose was also selected as suitable for enumeration of *L. paracasei* subsp. *paracasei* since it gave

excellent results compared with the reference method (Table 2).

MRS containing cysteine-HCl can provide optimal overall growth conditions for the non-selective enumeration of bifidobacteria (Roy, 2001; Leuschner, Bew, Simpson, Ross, & Stanton, 2003), and it was therefore selected as reference medium for *B. lactis*. Selective conditions for enumeration of this species were incubation at 45 °C, use of raffinose as a carbohydrate source and addition of 0.05% LiCl to suppress lactobacilli growth. The method was selective for *B. lactis* against the LAB strains studied. Overall characteristics of the method allowed reduction of the concentration of the antimicrobial compound LiCl to 0.05% instead of 0.2–0.3%, the amount usually added in selective media for enumeration of *Bifidobacteria* (Hartemink, Kok, Weenk, & Rombouts, 1996; Payne et al., 1999; Roy, 2001).

The Student's *t*-test was used to compare each species enumeration on both methods (reference and selective). The resulting *t* values (Table 2) were lower than the tabulated value of *t* (*t*_{tab} = 2.29) in all cases, which indicates that there were no significant differences between the two methods in bacterial enumeration at a probability level of 95%. The highest relative standard deviation of differences in counts between methods was observed for *L. delbrueckii* subsp. *bulgaricus*. In general, higher *L. delbrueckii* subsp. *bulgaricus* counts were found at 45 °C than at 37 °C (results not shown). Optimum temperature growth at 44 °C for *L. delbrueckii* subsp. *bulgaricus* has been previously reported (Beal, Louvet, & Corrieu, 1989), and a recommendation to increase temperature incubation for *L. delbrueckii* subsp. *bulgaricus* slowly growing strains is included in the ISO/FDIS 7889 IDF 117 standard on yoghurt colony count technique at 37 °C (ISO, 2002). Reproducibility of the methods was tested by the analysis of identical samples, acidified milk (pH 4.6) inoculated with each species at both high level (10⁷ cfu mL⁻¹) and low level (10⁵ cfu mL⁻¹), by two different operators and using different equipment. As shown in Table 2, the relative standard deviation of reproducibility was equal or lower than 0.14 log units.

Table 2

Counts (log cfu mL⁻¹) and evaluation of performance of the selective methods to enumerate *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis*

Parameter	<i>S. thermophilus</i>	<i>L. bulgaricus</i>	<i>L. acidophilus</i>	<i>L. paracasei</i>	<i>B. lactis</i>
Mean ^a (log cfu mL ⁻¹) of reference method	9.02	7.74	7.35	9.39	8.75
Mean ^a (log cfu mL ⁻¹) of selective method	9.01	8.04	7.34	9.41	8.78
Relative standard deviation of differences	0.08	0.47	0.08	0.10	0.12
Student's <i>t</i> ^b	0.70	1.58	0.53	0.52	0.76
Relative recovery	0.97	2.02	0.97	1.03	1.07
Relative standard deviation of reproducibility	0.14	0.13	0.13	0.07	0.08
Selectivity (%)	101.8	103.9	100.7	101.1	97.6

^aMeans are average from three independent analyses.

^b*t*_{tab} = 2.29.

To verify the selectivity of the methods, cultures of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis* were mixed at approximately 10^7 cfu mL $^{-1}$ in the ratio of 0.2, 1, 5, 0.2, and 1, respectively, and appropriate dilutions plated into the selective media. The results, expressed as the percentage of presumptive target counts in relation to theoretical counts, are shown in Table 2. Percentages close to 100% indicate that the selective methods yielded counts that were nearly equal to the theoretical counts, which indicates that the efficacy of the methods for selective enumeration of the five species in mixed cultures could be considered acceptable. Specificity and selectivity of the methods were also analysed in acidified milk, pH 4.6, that was inoculated with cultures of each target strain at low level (10^5 cfu mL $^{-1}$) and the other four strains at high level (10^7 cfu mL $^{-1}$). There was no interference between species for the enumeration of *S. thermophilus* and *B. lactis* in the corresponding selective media, and for *L. paracasei* subsp. *paracasei* when it was incubated in aerobiosis at 30 °C for 48 h in MRS agar. However, differential enumeration of *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus* in MRS-fructose (Fig. 1a) and of *L. acidophilus* and *L. paracasei* subsp. *paracasei* in MRS-maltose (Fig. 1b) could only be made when differences of counts between the two species were lower than 2 log units (results not shown).

3.2. Enumeration of bacteria in fermented milk products using the selective methods

The performance of the methods for selective enumeration of yoghurt and probiotic bacteria was carried out in the commercial probiotic product Simbiotic Drink (Priégola), containing *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis* as stated in the label, and the product manufactured with only the yoghurt bacteria. Results in Table 3 are averages from eight batches of each product analysed over 4 weeks. The results obtained indicated that there was low

variation between production batches, reliable counts for the probiotic and yoghurt strains and acceptable viability of the species throughout the shelf life of the products. The selective methods were therefore suitable for enumeration of the species, mostly because they were evaluated for the specific microorganisms present in the product. The results strengthen the rising opinion that selective or differential media should be evaluated for the specific strains of species of interest in each particular product (Lourens-Hattingh & Viljoen, 2001). As stated by Talwalkar and Kailasapathy (2004), the search for a single media in the literature that would provide reliable cell counts of *L. acidophilus*, *Bifidobacterium* spp., and *L. casei* in several different products could be unsuccessful.

Developed colonies (10%) from each selective media were subjected to confirmation test by species-specific PCR identification using primers based on the 16S rRNA gene sequence obtained from the LAB strains and on the partial sequence of the transaldolase gene sequence from *B. lactis* previously described (Requena et al., 2002). Specificity of the primers and PCR conditions to identify the analysed species were tested with DNA from pure cultures. Formation of specific amplicons was exclusively observed from the corresponding species (results not shown). All the presumptive target colonies analysed from selective and differential plates confirmed their identity by yielding PCR products of the expected sizes (Fig. 2). In addition, the species-specific primer pairs designed and the PCR conditions developed in this study proved to be a very rapid and effective method for the identification of the species.

3.3. Identification by PCR–DGGE of species present in the fermented milk

The primers used for species-specific PCR identification of colonies were also suitable for identification of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis* in culture-independent analysis of the fermented milk Simbiotic Drink using PCR–DGGE. The efficiency of separation was assayed by comparing amplicons obtained from pure cultures with the GC clamp attached to the forward or the reverse primer (see Material and methods). The appropriate products were combined to obtain the reference ladder that allowed for the identification of species in the fermented milk without prior isolation (Fig. 3). In spite of the length homogeneity of amplicons (Table 1 and Fig. 2), the technique allowed a distinguishable separation of fragments, showing a great detection and identification potential for analysis of these products. The efficiency of PCR–DGGE for lactobacilli and bifidobacteria identification of commercial probiotic products has been recently demonstrated (Fasoli et al., 2003; Temmerman, Scheirlinck, Huys, & Swings, 2003). In the present work, the high annealing temperature (60 °C) of the species-specific PCR applicable to the five species would have the additional advantage of carrying out one-step species identification by

Table 3
Viable counts of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis* in probiotic fermented milk and yoghurt through storage at 4 °C during four weeks

Species	Counts (cfu mL $^{-1}$) in fermented milk stored at 4 °C			
	1 wk	2 wk	3 wk	4 wk
Probiotic fermented milk				
<i>S. thermophilus</i>	9.32 (0.09)	9.15 (0.18)	9.22 (0.14)	9.14 (0.37)
<i>L. bulgaricus</i>	7.58 (0.28)	7.36 (0.53)	7.63 (0.07)	6.05 (1.03)
<i>L. acidophilus</i>	7.12 (0.30)	7.11 (0.33)	6.81 (0.33)	6.44 (0.36)
<i>L. paracasei</i>	6.49 (0.12)	6.48 (0.27)	6.49 (0.13)	6.47 (0.21)
<i>B. lactis</i>	8.08 (0.13)	8.03 (0.22)	7.97 (0.22)	8.15 (0.15)
Yoghurt				
<i>S. thermophilus</i>	8.88 (0.12)	8.91 (0.46)	8.98 (0.22)	8.94 (0.05)
<i>L. bulgaricus</i>	8.26 (0.11)	8.35 (0.13)	8.23 (0.13)	8.21 (0.07)

^aMeans are average from eight batches. Standard deviation in parenthesis.

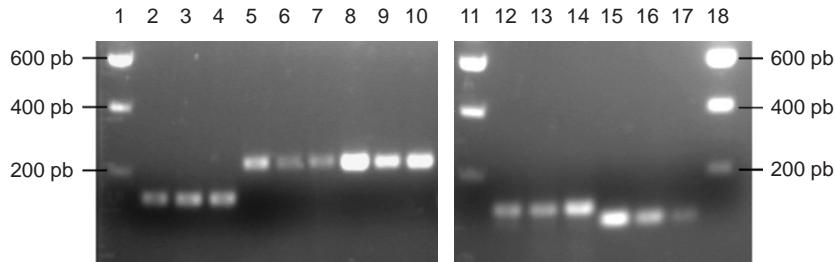


Fig. 2. Polymerase chain reaction products obtained from pure culture DNA and two colonies enumerated as *S. thermophilus* (lanes 2, 3 and 4), *L. delbrueckii* subsp. *bulgaricus* (lanes 5, 6 and 7), *L. acidophilus* (lanes 8, 9 and 10), *L. paracasei* subsp. *paracasei* (lanes 12, 13 and 14) and *B. lactis* (lanes 15, 16 and 17).

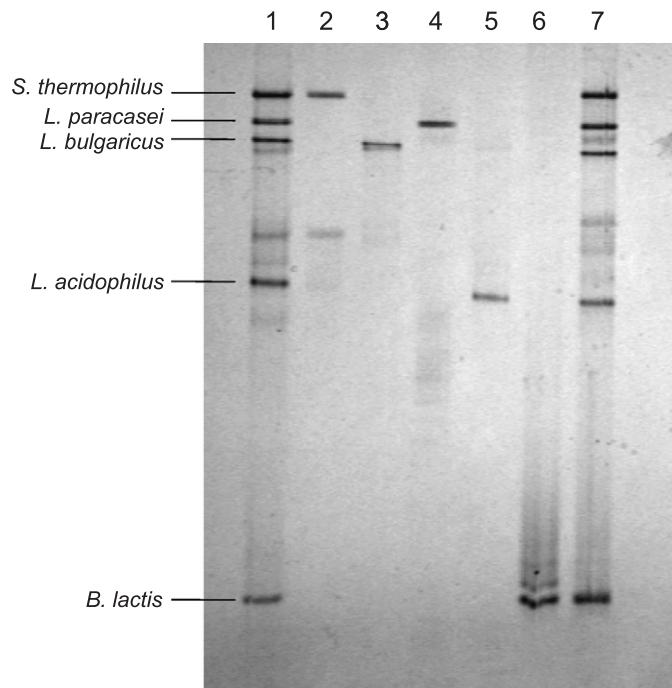


Fig. 3. Denaturing gradient gel electrophoresis (DGGE) analysis of the polymerase chain reaction products obtained from probiotic fermented milk using the species-specific primers for *S. thermophilus* (lane 2), *L. paracasei* subsp. *paracasei* (lane 3), *L. delbrueckii* subsp. *bulgaricus* (lane 4), *L. acidophilus* (lane 5) and *B. lactis* (lanes 6). Lanes 1 and 7: DGGE identification ladder.

multiplex PCR combined with separation of fragments by DGGE, a study that is currently underway.

4. Conclusions

The present study shows that the combined use of selective plating media and different incubation conditions provide an effective antibiotic-free approach to the selective enumeration of *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *B. lactis* in mixed cultures present in fermented milk products. The choice of methods was based on carbohydrate fermentation patterns and incubation at different temperatures and atmospheric conditions that were targeted to the species present in the product. Efficiency of the selective methods was verified by

evaluation of performance using statistical parameters such as precision, accuracy, reproducibility, selectivity and specificity, and by identification of the enumerated species by species-specific PCR. As a complementary advantage, this study also demonstrates that the combination of species-specific PCR and DGGE analysis shows a great detection and identification potential for verification of accurate species-labelling in fermented milk without previous isolation of the bacteria.

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Complete Genome Sequence of *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* SD96

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ABSTRACT The genome of *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* SD96, a strain used for cheese production, is presented. SD96 is refractory to phage attack, which is a desired property for starter bacteria. Its 10 plasmids provide industrially important traits, such as lactose and citrate metabolism, proteolytic activity, and phage resistance.

Lactococcus lactis, considered to be the model organism of lactic acid bacteria, has a long history of use in the dairy industry. Many mesophilic starter cultures used for cheese production contain several lactic acid bacteria, which are usually *Lactococcus* and *Leuconostoc* species (1). *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* is present in many mesophilic starter cultures and is responsible for a buttery aroma and gas formation from citrate. It has been shown that large genetic variety exists among lactococci, even between members of the same subspecies (2, 3).

Here, we present the whole genome of *L. lactis* subsp. *lactis* bv. *diacetylactis* SD96, a bacteriophage-insensitive mutant of strain SD16, which was originally isolated from an Italian cheese by starter culture provider Sacco S.r.l. To date, none of the phages contained in the comprehensive phage collection at Sacco S.r.l. has been found to be able to infect SD96, and dairies using SD96 have not reported any phage attack for this strain. Because of the contributions of plasmid-encoded genes to industrially relevant phenotypes, we were particularly interested in the plasmid content of this strain.

DNA for sequencing was isolated from an early-stationary-phase culture in M17 broth supplemented with 0.5% lactose, which was inoculated from a single colony. Total DNA was isolated using a genomic DNA purification kit (product number K0512) from Thermo Scientific. Sequencing was carried out on both PacBio Sequel and Illumina HiSeq 4000 platforms, using two distinct DNA preparations. Illumina sequencing using a PE150 PCR-free library provided 8.78 million raw paired-end 150-bp reads (SRA accession number [SRX6686433](#)). The sequences were processed and assembled using various software programs with default settings unless otherwise stated. Raw Illumina reads were processed using Geneious Prime (version 2019.1.3) (4). Briefly, adapter sequences and low-quality sequences were trimmed from both ends; finally, all reads above 50 bp were kept. This process resulted in 7.25 million sequences, with an average length of 138 bp. From the PacBio Sequel project using a 20-kb library, we received 331,855 raw reads, with an average length of 7,575 bp and a maximal read length of 44,814 bp (SRA accession number [SRX6711783](#)). For *de novo* assembly, the PacBio reads alone were processed and assembled using the Canu assembler (version 1.8) (5), which provided 13 linear scaffolds. The Circlator tool (version 1.5.5) (6) provided a closed chromosome and four plasmids. Another six plasmids were closed by identifying repeating regions on the scaffolds using the Repeat Finder plugin (version 1.0) in Geneious, followed by manual evaluation and trimming. Furthermore, the presence of replication-associated genes was confirmed for all 10 plasmids by sequence comparison with previously characterized *L. lactis* plasmids. Two scaffolds (4,944 and 5,967 bp)

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could not be closed or associated with the genome and did not contain replication-associated genes. Finally, the circularized sequences were polished using Pilon (version 1.23), for which the processed Illumina paired-end reads had been mapped to the circular sequences using Bowtie2 (version 2.3.0) in Geneious. The genome and plasmids were annotated using the RAST server (version 2.0) (7).

The 2.42-Mbp chromosome of SD96 has a GC content of 35.3%. The 10 plasmids have an average GC content of 34.4% (minimum, 30.3%; maximum, 36.5%) and sizes of 64.9, 49.6, 37.4, 25.4, 11.7, 8.3, 7.9, 7.3, 4.6, and 2.7 kbp (GenBank accession numbers [CP043518](#) to [CP043528](#)). Plasmids associated with protein degradation, lactose and citrate metabolism, and phage defense, as well as two cryptic plasmids with unknown purpose, were identified.

Data availability. The genome sequences of *L. lactis* subsp. *lactis* biovar diacetyl-lactis SD96, including its 10 plasmids, are available in GenBank under accession numbers [CP043523](#) (chromosome), [CP043525](#) (*pSD96_01*), [CP043526](#) (*pSD96_02*), [CP043527](#) (*pSD96_03*), [CP043528](#) (*pSD96_04*), [CP043524](#) (*pSD96_05*), [CP043518](#) (*pSD96_06*), [CP043519](#) (*pSD96_07*), [CP043520](#) (*pSD96_08*), [CP043521](#) (*pSD96_09*), and [CP043522](#) (*pSD96_10*). Raw data have been deposited in the Sequence Read Archive (SRA) under accession numbers [SRX6686433](#) (Illumina data) and [SRX6711783](#) (PacBio Sequel data).

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Chapter 2

***Bifidobacterium* transformation**

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Abstract

The protocol presented in this chapter describes a generic method for electro-transformation of *Bifidobacterium* spp., outlining a technique that is ideal for conferring selective properties onto strains as well as allowing the user to introduce or knock out/in selected genes for phenotypic characterisation purposes. We have generalized on the plasmid chosen for transformation and antibiotic selection marker, but the protocol is versatile in this respect and we are able to achieve transformation efficiencies up to 10^7 transformants/ μg of DNA.

Key Words Probiotic, bifidobacterial, genetic accessibility, electroporation

1. Introduction

Electroporation as a technique is based on the imposition of a strong electrical field to increase cell membrane permeability (1), thereby allowing the introduction of chemicals or nucleic acids (such as single and double stranded, circular or linear DNA) (2-4). Introduction of DNA into target cells facilitates their “transformation” into derivatives carrying or expressing a novel function, or mutants in which a target gene was removed or (re)introduced (5, 6). For microbiology the development and implementation of electroporation as a method of DNA introduction for genetic transformation purposes has been fundamental in the application of selective markers and the characterisation of hypothetical genes (5, 7).

Being Gram-positive, obligate anaerobes, particular members of the genus *Bifidobacterium* are purported to exert beneficial effects to their host and as a result a large body of research has been published by scientists who are working to better characterise these rather fastidious microbes, which are sometimes difficult to cultivate (8, 9). Members of the *Bifidobacterium* genus are ideal targets for genetic manipulation via electro-transformation, however, bifidobacteria are notoriously recalcitrant to genetic manipulation due to their extensive and diverse restriction/modification (RM) systems, thick cell wall, and sensitivity to oxygen (10-12). Only recently these hurdles have been investigated and overcome (5, 13, 14).

Here we describe a routine transformation methodology via electroporation for members of the genus *Bifidobacterium*. However, it should be noted that currently available literature suggests that the procedure for transformation may not be uniformly applicable for all

Bifidobacterium species and strains (13, 15). The protocol described below should therefore be used as an initial guide to achieve transformation, and modifications may thus have to be tested in order to suit each individual *Bifidobacterium* spp. For example, modifications can be made to the carbohydrate in the growth medium, plasmid to be transformed, amount of plasmid DNA used, electroporation parameters, and recovery medium.

2. Materials

2.1. Reagents:

1. Luria Bertani (LB) culture medium/agar: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride (and 16 g/L agar when required), autoclave to sterilize solution.
2. Modified de-Man-Rogosa-Sharpe (mMRS) Media: 10 g/L Tryptone (Peptone from Casein), 2.5 g/L yeast extract, 3 g/L tryptose, 3 g/L potassium phosphate dibasic (K_2HPO_4), 3 g/L potassium phosphate monobasic (KH_2PO_4), 2 g/L tri-ammonium citrate, 0.2 g/L pyruvic acid (sodium pyruvate), 0.575 g/L magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 0.12 g/L manganese (II) sulfate tetrahydrate ($MnSO_4 \cdot 4H_2O$), 0.034 g/L iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), dissolve all components in distilled water using a magnetic stirrer and then add 1 mL/L Tween80, autoclave to sterilize solution (See Note 1).
3. 38 g/L Reinforced Clostridium Medium (RCM; available as a premix from Oxoid), autoclave to sterilize solution.
4. 52.6 g/L Reinforced Clostridium Agar (RCA; available as a premix from Oxoid), autoclave to sterilize solution.
5. 10 % glucose solution: prepared in distilled water and 0.2 μ m filter sterilized. Store at 4°C and remake fresh weekly or as required.

6. 6 % L-cysteine-HCl solution: prepared in distilled water and 0.2 µm filter sterilized.
Store at 4°C and remake fresh weekly or as required.
7. Glycerol stock tubes: 200 µL 100 % glycerol aliquoted into 2 mL screw cap tubes and sterilized by autoclaving.
8. 80 % glycerol stocking solution for competent cells: prepared in distilled water, autoclave to sterilize solution.
9. Thermo Scientific GeneJET Plasmid Miniprep Kit (or equivalent).
10. Antibiotic for selection of specific plasmid, filter sterilized (e.g.: 5 µg/mL Chloramphenicol, final concentration)
11. Sucrose-citrate wash buffer: 0.21 g Citric Acid dissolved in 800 mL distilled water, adjust pH to 5.8 (using NaOH), make up to volume to 1 L with distilled water. Divide solution into five 200 mL bottles and add 0.5 M Sucrose, equivalent to 34.2 g per 200 mL bottle, autoclave to sterilize solution.
12. 1x TAE buffer: 4.844 g/L Tris base, 1.21 mL/L acetic acid, and 0.372 g/L EDTA.
13. 1 % agarose dissolved in TAE by microwaving.

2.2. Equipment:

1. Nanodrop1000/Qubit (DNA quantification equivalent)
2. 1.5 mL tubes
3. 50 mL falcon tubes
4. 25 mL serological pipettes
5. Microcentrifuge
6. Gel-electrophoresis system
7. Transilluminator for gel imaging
8. Anaerobic work station (10 % hydrogen, 10 % carbon dioxide and 80 % nitrogen)

9. Refrigerated centrifuge with rotor for 50 mL falcon tubes
10. Electroporator and electroporation cuvettes (2 mm)
11. Spectrophotometer measuring optical density at a wavelength of 600 nm

3. Methods

1. Recover desired plasmid from relevant bacterial host (e.g.: *Escherichia coli*, see Note 2) achieved with Plasmid Miniprep Kit following the manufacturer's instructions.
2. Plasmid recovery can be confirmed by standard agarose gel electrophoresis.
3. Quantify extracted plasmid DNA using spectrophotometric methods (e.g.: Nanodrop or Qubit).
4. To prepare bifidobacterial competent cells, overnight cultures are first prepared in 10 mL RCM supplemented with 0.05 % L-cysteine stock solution (with additional carbohydrate if required- strain specific). Incubate cultures at 37°C anaerobically overnight (~16 hours) without shaking.
5. The following day inoculate 5 mL of the overnight culture into 40 mL of mMRS, with 1 % vol/vol addition of filter sterilised stock sugar (e.g.: glucose) and 0.05 % L-cysteine stock solution.
6. Incubate anaerobically at 37°C until optical density (OD_{600nm}) reaches 0.6-0.9 (~3 hours), monitor OD_{600nm} with a spectrophotometer by aseptically removing 1 mL of growing culture approximately every 1-2 hours.
7. Once an OD_{600nm} of 0.6-0.9 is reached, place cultures on ice for 20 minutes, inverting every 5 minutes (see Note 3).
8. Harvest cells by centrifugation: 4°C, 4052 x g for 10 min.
9. Discard supernatant into waste.

10. Wash cells with ice cold 25 mL sucrose-citrate wash buffer. To resuspend the cell pellet use the serological pipette to knock the pellet off the side of the tube after adding the ice cold buffer and gently mix.
11. Repeat steps 8-10.
12. During centrifugation, ensure that recovery medium RCM is pre-warmed to 37°C.
13. Prepare 1.5 mL tubes with aliquoted plasmid DNA (concentration can vary, we recommend starting with 200 ng) and label electroporation cuvettes, these should be chilled on ice before use.
14. After final wash and centrifugation of bifidobacterial cells, discard supernatant into waste.
15. Gently resuspend cells in 200 µL sucrose-citrate wash buffer. If freezing competent cells, add 200 µl of 80 % glycerol (see Note 4), dispense into pre-chilled labelled 1.5 mL tubes and store at -80°C.
16. Mix 50 µL of competent cells and plasmid DNA (e.g.: 200 ng), transfer total volume to an electroporation cuvette.
17. Prepare a negative control for electroporation by only adding 50 µL of competent cells to a cuvette.
18. Carry out electro-transformation as quickly as possible, using the following settings:
 - 25 µF
 - 200 Ohms
 - 2000 V
19. Following electroporation, resuspend cells in cuvette to a final volume of 1 mL with prewarmed RCM and incubate at 37°C anaerobically for 1 hour.

20. Plate 100 µL of transformed cells onto RCA (plating dilutions of the cell preparation are also recommended, e.g.: 10^{-1} , 10^{-2} and 10^{-3}) with appropriate selective antibiotic (e.g.: final concentration 5 µg/mL chloramphenicol).

21. Incubate plates anaerobically at 37°C for 2-3 days.

22. Colony counts can then be performed to determine the transformation efficiency.

Transformation efficiency = number of colonies counted on plate / (μg plasmid DNA transformed / total dilution of DNA before plating)

4. Notes

1. Improved transformation efficiency has been observed when mMRS is 0.2 µm filter sterilized in comparison to autoclave sterilization. Filter sterilized medium should be stored at 4°C and remade fresh weekly or aliquoted and frozen at -30°C.

2. Suggested plasmids for bifidobacterial transformation are listed below in Table 1 (NB. See also chapter 15 of this book for information on plasmids that replicate in bifidobacteria). It should be noted that one of the biggest hurdles for successful transformation is resident restriction-modification (RM) systems (10, 16). Selection of a plasmid with fewer RM motifs (strain-specific) can drastically improve the recovery of transformants. Alternatively, a plasmid can be methylated chemically (eg: NEB, GpC Methyltransferase (M.CviPI)) or by first transforming a given plasmid into a methylase-positive strain such as *E. coli* EC101 (DAM+; methylates GATC sites) otherwise an *E. coli* strain in which a bifidobacterial methylase gene is expressed (16-19). Transformation of EC101 and other *E. coli* strains are performed using methods published by Dower, Miller (20).

Table 1. Example plasmids for *Bifidobacterium* transformation

Plasmid	Relevant characteristics	Citation
pNZ8048	Cm ^R , pSH71 replicon, inducible nisA promoter	(21, 22)
pNZ44	Cm ^R , pNZ8048 containing constitutive P44 promoter from <i>L. lactis</i>	(23)
pPKCM	Cm ^R , Set of <i>E. coli-Bifidobacterium</i> shuttle vectors based on pBlueCm	(24)
pSKEm	Em ^R , <i>E. coli-Bifidobacterium</i> shuttle vector derived from pErythromycin	(24)
pAM5	Tet ^R , pBC1-pUC19 [tet(W)]	(25)

3. When preparing competent cells ensure that they are always kept chilled (on ice) to ensure cells remain receptive to plasmid DNA. This includes making the wash buffer at least the day before, storing at 4°C overnight and then keeping on ice during the cell wash steps.

4. Improved transformation efficiency is always observed when freshly made competent cells are used. Use of frozen competent cells is still possible for electroporation but expect a decrease in transformation efficiency.

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Production of Nisin Z by *Lactococcus lactis* Isolated from Dahi

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Abstract *Lactococcus lactis* CM1, an isolate from homemade “Dahi,” a traditional fermented milk from India, used maltose as carbon source to produce a high level of bacteriocin. The bacterial cell mass and the bacteriocin production correlated with the initial pH of the medium and were highest when the initial pH was 11.0. The level of bacteriocin reached its peak at the late log phase with concomitant reduction of culture pH to 4.2, regardless of the initial pH of the medium. A combination of maltose and an initial medium pH of 11 resulted in the highest bacteriocin production. The antibacterial spectrum of the bacteriocin was closely similar to that of nisin and it inhibited a number of food spoilage and pathogenic bacteria. Upon sodium dodecyl sulfate polyacrylamide gel electrophoresis, the compound migrated close to the position of nisin (3.5 kDa). However, it had higher stability than nisin at a wide range of pH and temperature. PCR amplification using nisin gene-specific primers and sequencing of the amplified DNA revealed the structural gene for the bacteriocin to be identical to that of *nizZ*.

Keywords *Lactococcus lactis* · Nisin · Inhibitory spectrum

Introduction

Lactic acid bacteria (LAB) are of great economic value for fermentation. They are extensively used in the production of various fermented foods like yogurt, cheese, buttermilk, and sausage. Many LAB produce bacteriocins, which are extracellular bioactive proteins with antibacterial activity towards species related to producer bacteria and a wide variety of Gram-positive food-borne pathogens and spoilage microorganisms [1, 2]. The

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bacteriocins produced by LAB have an important role as biopreservatives due to increasing consumer awareness of the potential risks derived not only from food-borne pathogens, but also from the chemical preservatives used to control them. Among the bacteriocins, nisin is the most extensively studied bacteriocin and is a classical example of a biopreservative.

The bacteriocin nisin, produced by certain strains of *Lactococcus lactis*, is nontoxic [3]. It is approved for use in the USA, the UK, and the European Union [4], and is currently recognized as a generally-regarded-as-safe (GRAS) food additive and preservative mainly for processed foods in approximately 50 countries worldwide. Nisin is a ribosomally synthesized, low-molecular-weight, heat-stable polycyclic peptide with 34 amino acid residues and has two natural variants, nisin A and Z. Nisin Z differs from nisin A by a single substitution at position 27, with asparagine (nisin Z) in place of histidine (nisin A) [5]. This structural modification of nisin Z imparts higher solubility and diffusion characteristics, which are important for food application [6]. Although *L. lactis* has been reported to grow at pH 9 [7], in general, LAB, including *L. lactis*, are grown in media of pH 6 to 7 for the production of bacteriocin. However, in these cultures, as the pH decreases to a certain minimum due to lactic acid accumulation, the growth of the organism, as well as of bacteriocin, stops [8].

The search for new strains of LAB able to grow at high pH for the production of increased levels of bacteriocin or for new or natural variants of bacteriocin is of immense significance to increase the current range of biopreservative applications. In this regard, many nisin-producing strains of *L. lactis* have been isolated from both dairy and nondairy traditional fermented foods [9–12]. However, the characterization of nisin from isolates of LAB from “Dahi,” a traditional fermented milk from India (a product similar to yogurt), has not been done [13–15]. In this paper, we report high production of nisin Z by *L. lactis* strain CM1 isolated from Dahi under optimized conditions and compare it with that of nisin production by the standard strain *L. lactis*, American Type Culture Collection (ATCC) 11454. The biochemical properties and the antibacterial spectrum of the bacteriocin produced by the strain CM1 have also been investigated.

Materials and Methods

Cultures and Growth Conditions

Bacterial strains used as indicators are *Lactobacillus plantarum* NCDO 955, *Pediococcus acidilactici* LB42, *Leuconostoc mesenteroides* Ly, and *Enterococcus faecalis* MB1. These are sensitive to most bacteriocins and are able to grow at pH 4.0 and above. B. Ray, Department of Animal Science, University of Wyoming, USA, provided these strains along with *P. acidilactici* F⁺. Lactic acid bacteria were grown in tryptone–glucose–yeast extract (all at 1%, TGE) medium [16] at 37°C. *Listeria* sp. was grown at 37°C in brain heart infusion (Hi Media, Mumbai, India), *Salmonella* sp., *Vibrio* sp., *Staphylococcus* sp., *Bacillus* sp., *Geobacillus* sp., *Enterobacter* sp., and *Escherichia coli* in Luria Bertani broth and *Clostridium* sp. were grown in reinforced clostridial medium. *Geobacillus* was grown at 60°C. A nisin-producing strain, *L. lactis* ATCC 11454, was used for comparative studies. The effect of different carbon sources on growth and bacteriocin production by *L. lactis* was studied in tryptone–yeast extract (TE; both at 1%) medium (Table 1). All the strains with ATCC and Microbial Type Culture Collection (MTCC) numbers used in this study (Table 2) were procured from MTCC, Institute of Microbial Technology, Chandigarh, India. *Geobacillus thermoleovorans*, *Vibrio cholerae*, *Staphylococcus aureus*, strains of *E. coli*,

Table 1 Influence of carbon source on growth (OD_{650}) and bacteriocin production (AU/ml) of *L. lactis* CM1 and *L. lactis* ATCC 11454 at 37°C.

Substrate	CM1		ATCC 11454	
	OD_{650}	AU/ml	OD_{650}	AU/ml
Glucose	1.12	2,000	0.96	2,000
Xylose	1.1	2,400	0.22	400
Sucrose	1.42	2,400	1.02	3,000
Lactose	1.12	2,000	0.18	200
Galactose	1.1	2,000	0.94	3,000
Maltose	1.2	3,000	0.80	2,000
Mannose	1.14	2,000	1.02	2,000
Trehalose	1.74	2,800	1.30	3,000
Cellobiose	1.8	1,000	1.02	2,000

Each carbon source was added at 1% to tryptone–yeast extract medium, pH 6.5. Fermentation was carried out for 7 h, except for growth and bacteriocin yield of ATCC 11454 on cellobiose and galactose for 24 h.

and *Pseudomonas putida* were from our laboratory collection. All the cultures were stored at –20°C with 10% dimethyl sulfoxide.

Isolation of Bacteriocin-Producing Strain

Samples of homemade Dahi prepared by fermenting cow milk at 37°C for 24 h by its natural microflora were serially diluted, and an aliquot of each dilution was plated on TGE-agar

Table 2 Antibacterial spectrum of bacteriocin of *L. lactis* CM1 and *L. lactis* ATCC 11454.

Target strain	<i>L. lactis</i> CM1	<i>L. lactis</i> ATCC 11454
<i>Lactobacillus acidophilus</i> ATCC 4356	–	–
<i>Lactobacillus delbrueckii</i> ATCC 4797	+	+
<i>Lactobacillus plantarum</i> NCDO 955	+	+
<i>Lactobacillus rhamnosus</i> ATCC 7469	+	+
<i>Lactococcus lactis</i> ATCC 19257	+	+
<i>Pediococcus acidilactici</i> LB 42	+	+
<i>Pediococcus acidilactici</i> F ⁺	+	–
<i>Bacillus cereus</i> MTCC 1272	+	+
<i>Geobacillus thermoleovorans</i>	–	–
<i>Clostridium perfringens</i> ATCC 3624	+	+
<i>Enterococcus faecalis</i> MB1	+	+
<i>Leuconostoc mesenteroides</i> Ly	+	+
<i>Leuconostoc mesenteroides</i> ATCC 10830	+	+
<i>Listeria monocytogenes</i> ATCC 19111	+	+
<i>Staphylococcus aureus</i>	+	+
<i>Escherichia coli</i> EPEC	–	–
<i>Escherichia coli</i> ETEC	–	–
<i>Enterobacter aerogenes</i> ATCC 13048	–	–
<i>Pseudomonas putida</i>	–	–
<i>Salmonella typhimurium</i> ATCC 23565	–	–
<i>Vibrio cholerae</i>	–	–

Lactococcus lactis ATCC 11454 was used as positive control. Plus signs represent zone of inhibition (15 mm or more in diameter).

medium. The plates were incubated at 37°C. The colonies were examined for bacteriocin activity by the spot-on-the-lawn assay method against the four indicator strains [17]. The lawn was made by overlaying approximately 10^6 cells in TGE soft agar from each colony onto the TGE plate. The isolates showing zone of growth inhibition of all four indicator strains were streaked several times on TGE plates to obtain pure cultures and were further examined for bacteriocin production in liquid medium by a method described previously [16]. Cell-free culture supernatant of each isolate was divided into two parts: one part was boiled and neutralized and the other part was only boiled but not neutralized. Both samples were spotted on a TGE agar plate overlaid with the indicator strain, *L. plantarum* NCDO 955. Following incubation at 37°C, the plates were observed for zone of growth inhibition. For a comparison, the culture supernatants of *P. acidilactici* F⁺ and *L. lactis* ATCC 11454 were used during the isolation procedure. The experiment was replicated thrice.

Identification of Bacteriocin-Producing Strain

The bacteriocin-producing isolate was identified by its colony morphology, Gram-staining, and biochemical tests [18, 19], as well as by 16S rRNA gene sequencing [20]. The isolate was examined for acid production from 49 compounds as sole carbon source using API 50 CH Test kit (BioMerieux, Crappone, France) according to the manufacturer's instructions.

DNA Manipulation, PCR, and DNA Sequencing

Genomic DNA of the bacteriocin-producing strain was isolated by lysozyme–proteinase K procedure [21]. Approximately 50 ng of the DNA was used as template for PCR amplification of 16S rRNA or of nisin gene in a total volume of 50 μl containing 1.25 U of *pfu* polymerase (Fermentas, Hanover, MD, USA), 40 pmol of each primer, 200 μM of each deoxynucleoside triphosphate, and 2 mM MgCl₂. The 16S rRNA gene was amplified in 35 cycles with a thermocycler (Perkin Elmer, Waltham, MA, USA); each cycle consisted of a denaturing step at 95°C for 1 min, a primer annealing step at 60°C for 1 min, and an extension step at 72°C for 5 min. Bacteria-specific universal primers used for amplification of 16S rRNA gene were the forward primer 27F (5'-AGAGTTTGATCATGGCTC-3') and the reverse primer 1327R (5'-CTAGCGATTCCGACTTCA-3') [20]. The primer pair amplifies a 1.3-kb fragment of DNA. The PCR amplification conditions for nisin gene were similar to that for 16S rRNA gene, except that the primer annealing temperature was 53°C. Two sets of primers were used for nisin gene amplification. These were (1) PNIS1 (5'-CGCGAGCATA ATAAACGGCT-3') and PNIS2 (5'-GGATAGTATCCATGTCTGAAC-3'), complementary to regions 80 bp upstream and 29 bp downstream of the coding region of the *nisA* and *nisZ* genes, respectively [5], and (2) NS1 (5'-TAGATACAATGATTTCGTTTC-3') and NS2 (5'-AGCTCACTACTATTATGGT-3') complementary to nucleotide positions 106–125 upstream and positions 919–938 downstream of the start codon of *nis A* and *nis B* genes, respectively [22]. PCR products were checked by electrophoresis on 1.5% agarose gel. A 100-bp DNA ladder was used as the molecular marker (Fermentas). PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and sequenced from both ends with an ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA) using the same oligonucleotide primers used for PCR. All the primers were synthesized by Genei, Bangalore, India.

Production and Assay of Bacteriocin

Kinetics of growth and bacteriocin production was studied at 37°C in TGE medium for 10 h from a 2% inoculum of an overnight culture of *L. lactis* CM1. Bacterial growth was

measured following the OD of the culture at 650 nm, whereas bacteriocin activity in the culture supernatant was measured by the extinction-to-the-dilution method [16]. The culture broth was withdrawn periodically and treated at 100°C for 10 min. An aliquot of the treated sample was serially diluted (1:2 to 1:50) with deionized water, and 5 µl from each dilution was spotted on the TGE agar plate overlaid with soft agar (0.7%) containing 10^6 cells of an overnight grown *L. plantarum* NCDO 955. The plates were incubated at 37°C and examined for any zone of growth inhibition. The activity unit per milliliter (AU/ml) was defined as the reciprocal of the highest dilution that produced a definite zone of inhibition. For a comparison, a growth kinetics study of *L. lactis* ATCC 11454 with CM1 was performed in parallel. All the experiments were performed in triplicate.

To study bacteriocin production using different carbohydrates, CM1 was grown overnight in TGE medium at 37°C. The cells were harvested by centrifugation at 10,000 rpm for 10 min, washed twice with saline, and resuspended in TE broth to its original density. This was used to inoculate fresh TE medium supplemented with different carbohydrates to a final concentration of 1%. The pH of each medium was adjusted to 6.5 before inoculation. Following incubation at 37°C, the growth and bacteriocin activity were measured as described. A nisin-producing strain *L. lactis* ATCC 11454 was also grown for comparison. The experiment was done in triplicate.

In a separate experiment, bacteriocin production was studied in TGE medium adjusted to various pH values ranging from 5.0 to 11.0. The strain *L. lactis* CM1 grown overnight in TGE medium at 37°C was harvested by centrifugation at 10,000 rpm for 10 min, washed with saline, and finally resuspended in TGE medium to its original density. The media with different pH values were inoculated with this inoculum at 2%. The fermentation was carried out at 37°C for 24 h. The activity of bacteriocin produced was assessed. The *L. lactis* ATCC 11454 was also grown under the same conditions for comparison. Following inoculation, the initial pH of the media was checked in a duplicate set. The experiment was replicated thrice.

Stability of Bacteriocin

To determine the thermostability of the bacteriocin at different pH values, it was precipitated at 70% saturation of ammonium sulfate, dialyzed, and adjusted to the desired pH in the range of 2.0 to 12.0 using 1 M NaOH or 1 M HCl. The preparations were then incubated either at 37°C for 5 h, at 100°C for 1 h, or at 121°C for 15 min, and then readjusted to pH 6.0. All the samples were then assayed to determine the residual activity using the indicator strain, *L. plantarum* NCDO 955. Untreated sample or buffer alone served as the control. Nisin A produced by *L. lactis* ATCC 11454 was also treated similarly, and its stability was determined for a comparison. For stability determination, the preparations contained 2,000 AU/ml of the bacteriocin activity. The effect of sodium dodecyl sulfate (SDS), urea, Tween 80 or Triton X-100 at 1%, and EDTA (10 mM) or mercaptoethanol (50 mM) on the activity of bacteriocin was studied at 37°C for 5 h. Bacteriocin preparation was mixed with each of the chemicals, and after incubation, the samples were diluted 10-fold and a 5-µl aliquot was spotted on a TGE agar plate overlaid with *L. plantarum* NCDO 955. The plates were incubated at 37°C for 24 h and observed for any zone of growth inhibition. Untreated samples and the chemicals served as the control. In a separate experiment, the culture supernatants adjusted to pH 6.5 were treated with protease, namely, trypsin, papain, or proteinase K, at 37°C for 2 h, and the residual activity was determined to assess the stability of the bacteriocin to these treatments. The untreated culture supernatant served as the control. Nisin A of *L. lactis* ATCC 11454 was used for

comparison under the same experimental conditions. The experiments were done in triplicate.

Antibacterial Activity Spectrum

The strain CM1 was grown in TGE medium at 37°C for 24 h. Semipurified bacteriocin was prepared by 70% ammonium sulfate precipitation of the heat-treated cell-free culture supernatant and was examined against Gram-positive and Gram-negative bacteria (Table 2). A 50- μ l aliquot of the bacteriocin preparation corresponding to 2,000 AU/ml was spotted on the lawn of the target bacteria prepared using 10⁶ of the overnight grown cells. In a parallel experiment, similarly prepared nisin from the culture supernatant of *L. lactis* ATCC 11454 was used for a comparison. The plates were incubated at 37°C for 24 h, and at 60°C for *Geobacillus* sp. Following incubation, the plates were observed for any zone of growth inhibition. Each assay was replicated thrice.

Detection of Bacteriocin Activity and Molecular Size Approximation by SDS Polyacrylamide Gel Electrophoresis

Bacteriocin activity was directly detected on polyacrylamide gel (15%) as described previously [23]. The isolate was grown in a medium containing glucose (1%) and yeast extract (1%), but without tryptone. Bacteriocins were partially purified from the culture supernatants by CM-cellulose chromatography and were electrophoresed at 200 V for 3 h. After electrophoresis, the gel was cut vertically into two halves. One half was stained with silver nitrate to visualize the protein bands [24]. The other half was washed in sterile deionized water for 3 h at room temperature to remove SDS and then overlaid with soft agar containing 10⁶ cells of an overnight grown culture of *L. plantarum* NCDO 955. After incubation at 30°C for 16 h, it was examined for zone of growth inhibition. The protein band corresponding to the antibacterial activity was identified. Low-range standard peptides were used as molecular weight markers (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for size approximation.

Results

Out of a total of 616 colonies isolated from Dahi sources, the isolate CM1 produced a zone of growth inhibition against all the four bacteriocin-sensitive indicator strains. The boiled culture supernatant of the isolate, whether neutralized or not, exhibited similar inhibitory activity. The inhibitory activity was destroyed by treatment with proteases, namely, trypsin, papain, and proteinase K, indicating that the isolate released a heat-stable proteinaceous substance into the growth medium.

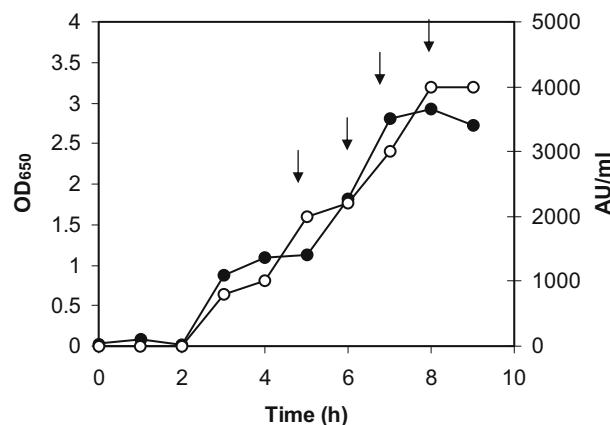
Scanning electron microscopy demonstrated the cells of the isolate CM1 to be coccoid and 1 μ m in diameter. The bacterium is Gram-positive, catalase-negative, VP-positive, and able to hydrolyze arginine. The isolate could grow at 10°C but could not at 45°C or at 6.5% NaCl. It did not produce gas from glucose. The results indicated that the strain belonged to the genus *Lactococcus* [18]. The isolate fermented ribose, mannitol, arbutin, salicin, cellobiose, maltose, lactose, esculin, trehalose, and β -gentibiose, but not sorbitol, melibiose, inulin, melezitose, turanose, tagatose, or raffinose, similar to *L. lactis* subsp. *lactis* [25]. In contrast to *L. lactis* subsp. *lactis* ATCC 19435, the isolate, however, utilized L-arabinose, sucrose, amygdalin, and D-xylose. On the basis of acid production from 49 carbohydrates,

the isolate was tentatively identified as *L. lactis* [19]. The strain was further characterized by sequencing of its PCR-amplified 16S rRNA gene. Comparison of the 16S rRNA gene sequence of the strain CM1 with those in the database showed 99% homology to those of *L. lactis*, and having the highest level of identity with that of *L. lactis* subsp. *lactis*.

The kinetics of growth and bacteriocin production of the strain CM1 was studied in media of various pH ranging from 5.0 to 11.0 and at temperatures ranging from 25° to 40°C. The optimum temperature for growth and bacteriocin production of the strain was a broad one from 30° to 37°C. However, at 40°C, both bacteriocin production and growth were adversely affected. At 37°C, bacteriocin production was detected at the early log phase of growth and the level increased in parallel to growth of the bacterium and reached its maximum at the late log phase, and thereafter, it remained essentially constant. Concomitant to growth, the pH of the culture gradually declined to 4.2. Although bacteriocin production and cell mass increased with an increase in the initial pH of the growth medium, the final pH of the cultures was always lowered to 4.2 regardless of the initial pH. The highest level of bacteriocin production by the strain was assessed to be 2,000 AU/ml in the TGE medium, with an initial pH of 6.5 and was equal to that of *L. lactis* ATCC 11454. Although growth of the strain CM1 was recorded to be highest (1.8 OD₆₅₀) with cellobiose, the bacteriocin production was lowest with this carbon source. On the other hand, the highest level of bacteriocin (3,000 AU/ml) of the strain CM1 was produced with maltose, whereas trehalose was the next preferred carbon source for the bacteriocin production (Table 1). As compared, the strain ATCC 11454 produced the highest level of bacteriocin with sucrose, galactose, and trehalose. Xylose and lactose supported only a little growth and bacteriocin production of the strain ATCC 11454 in contrast to the strain CM1, where a high level of growth and bacteriocin production was observed with these three carbon sources (Table 1).

The effect of culture pH adjustment on growth and bacteriocin production of *L. lactis* strain CM1 was studied. In TGE medium, pH 6.5, where glucose served as the carbon source, the strain CM1 attended only an OD₆₅₀ of 1.12, and the bacteriocin production was 2,000 AU/ml (Fig. 1) when the medium pH was down to 4.2. However, if the medium pH was adjusted back to pH 6.5 with the addition of alkali at this time, further growth and bacteriocin production was observed. Figure 1 shows that the addition of alkali to the culture at the fifth, sixth, and seventh hours back to pH 6.5 allowed further growth and bacteriocin production. However, at the eighth hour, such pH adjustment yielded no further

Fig. 1 Effect of pH adjustment on the production of cell mass (closed circles) and bacteriocin (open circles) of *L. lactis* CM1 in TGE medium at 37°C. The initial pH of the medium was 6.5. Arrows indicate the time points (5, 6, 7, and 8 h) when alkali was added to adjust the pH back to 6.5



growth or bacteriocin production, and the final growth attended was almost 3 OD₆₅₀ and the bacteriocin production was almost 4,000 AU/ml (Fig. 1).

The effect of the initial medium pH on growth and bacteriocin production of *L. lactis* strains CM1 and ATCC 11454 was studied. For this, the pH of the TGE medium was initially adjusted to different values ranging from 6.5 to 11.0, and the growth and bacteriocin production were assessed (Fig. 2). It was found that both growth and bacteriocin production of the strains increased almost linearly up to pH 9. Further increase in growth and bacteriocin production was noted for the strain CM1 at initial medium pH values of 10 and 11; the strain ATCC 11454, however, failed to grow and thereby to produce bacteriocin at an initial medium pH of 10. At an initial medium pH of 11, the growth of the strain CM1 almost reached an OD₆₅₀ of 2.5, and the level of bacteriocin was almost 5,000 AU/ml (Fig. 2).

In a separate experiment, the glucose of the TGE medium was replaced with maltose as the carbon source, the initial pH of the medium was either adjusted to 6.5 or to 11.0, and the kinetics of growth and bacteriocin production of *L. lactis* strain CM1 were studied (Fig. 3). It was observed that, in the medium with an initial pH of 6.5, the strain grew to an OD₆₅₀ of 1.2, and the bacteriocin production of the strain was about 3,000 AU/ml. These values compared to be only about one-third to those attended in the medium with an initial pH of 11 where the growth of the strain was higher than 3 OD₆₅₀ and the bacteriocin production was almost 9,000 AU/ml (Fig. 3).

At 37°C, the activity of the bacteriocin produced by the strain CM1 was found to remain stable for at least 5 h at pH values between 2.0 to 9.0 and lost only half of the activity at pH 10.0 and 90% at pH 12.0 (Fig. 4). The activity of nisin A produced by the strain ATCC 11454, however, was found to be stable in the pH range of 2.0 to 6.0, and it lost 50% of its activity at pH 7.0 and 100% at pH 12.0 (data not shown). At an acidic pH (2.0 to 4.0), the activity of bacteriocin produced by the strain CM1 remained stable even at a high temperature of 121°C. Treatment of the bacteriocin obtained from the strain CM1 for 1 h at 100°C caused a loss of only 20% of its activity at pH 6.0 (Fig. 4), in contrast to the loss of 80% of nisin A activity of ATCC 11454. The antibacterial activity was fully retained following exposure to SDS, Tween 80, Triton X100, urea, EDTA, and mercaptoethanol for 5 h at 37°C.

Fig. 2 Effect of initial medium pH on growth (closed circles, *L. lactis* CM1; closed squares, *L. lactis* ATCC 11454) and bacteriocin activity (open circles, *L. lactis* CM1; open squares, *L. lactis* ATCC 11454)

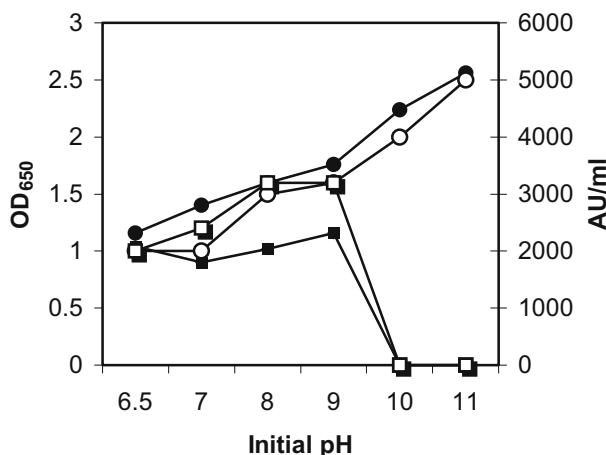
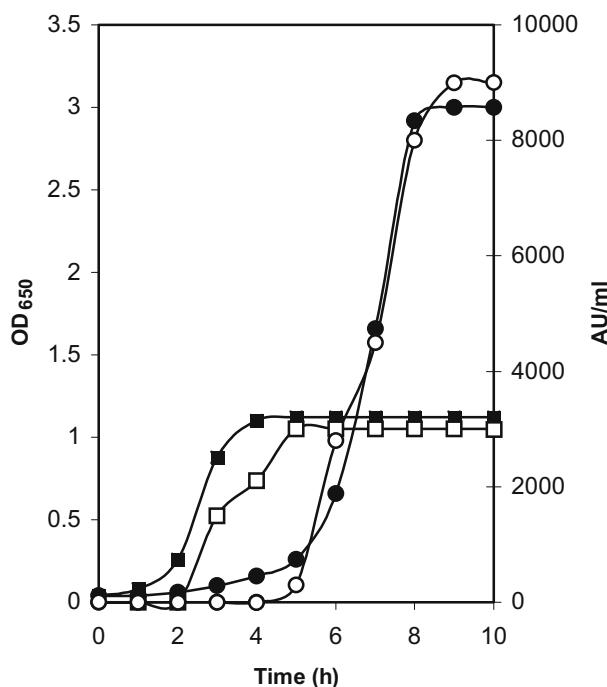


Fig. 3 Growth and bacteriocin activity of *L. lactis* CM1 in maltose containing medium with different initial pH. Growth was recorded in media with an initial pH of 6.5 (closed squares) and 11.0 (closed circles) and bacteriocin activity in media with an initial pH of 6.5 (open squares) and 11.0 (open circles)



The bacteriocin exhibited a broad antibacterial spectrum against several food spoilage and pathogenic bacteria, as well as related species of LAB. However, it was not effective against Gram-negative bacteria, but proved to be active against *Bacillus cereus*, *Listeria monocytogenes*, *Clostridium perfringens*, *E. faecalis*, *S. aureus*, and several species of LAB (Table 2). Additionally, *P. acidilactici* F⁺ resistant to nisin A of ATCC 11454 was killed by the bacteriocin produced by CM1.

Fig. 4 Effect of temperature on the stability of the bacteriocin of *L. lactis* CM1. Circles, 37°C; squares, 100°C; triangles, 121°C

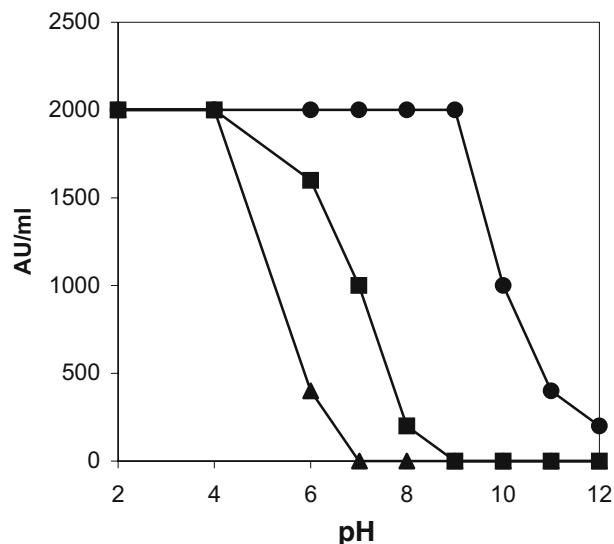
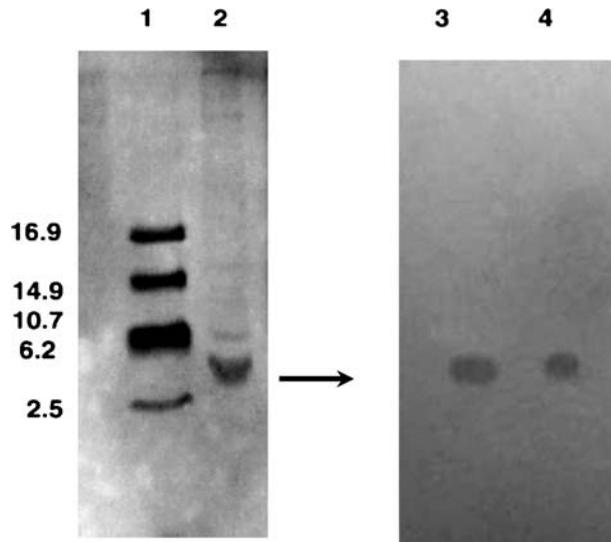


Fig. 5 Antimicrobial activity of partially purified bacteriocin of *L. lactis* CM1 following SDS polyacrylamide gel electrophoresis. Lane 1, low-molecular-weight protein markers; lane 2, silver-stained band of bacteriocin; lane 3, zone of inhibition of *L. plantarum* NCDO 955 by the nisin Z of *L. lactis* CM1; lane 4, zone of inhibition of *L. plantarum* NCDO 955 by the nisin A of *L. lactis* subsp. *lactis* ATCC 11454



The bacteriocin produced by the strain CM1 was found to be a short peptide (about 3.5 kDa), as judged by SDS polyacrylamide gel electrophoresis (Fig. 5). The molecular weight was judged by comparing the distance of the zone of growth inhibition of the indicator strain, *L. plantarum* NCDO 955 overlaid onto the gel from the point of application with the distances traversed by standard proteins.

The presence of nisin gene in the strain CM1 was identified by PCR amplification using two sets of nisin gene-specific primers and sequencing of the amplified products. The PCR product with each set of primers upon agarose gel electrophoresis showed a single band of expected size and were 330- and 835-bp fragments, respectively, for the sets of PNIS and NS primer pairs. The DNA sequence of the PCR amplicons was found to be identical to the *nizZ* sequences in the database. The result indicates that the bacteriocin produced by *L. lactis* CM1 is nisin Z. However, the sequence corresponding to the *nizB* gene, which is involved in dehydration of serine and threonine causing posttranslational modification of nisin [26], differed by one nucleotide out of 678 nucleotides sequenced in both orientations. It was found that this change in one nucleotide added a different codon for the same amino acid.

Discussion

Nisin is produced by certain strains of *L. lactis*. The success of the practical application of nisin as a biopreservative would rely on overcoming the problems associated with its low level of production and, thereby, would result in its high cost. A number of attempts were made to produce high levels of bacteriocin using different cumbersome processes. These involved either the addition of alkali to culture following cessation of growth, extraction of lactate, or removal of lactate by microfiltration or through assimilation of lactate by the yeast *Kluyveromyces marxianus* [27, 28]. Readjustment of culture pH was found to extend the growth period and, thereby, enhance the yield of bacteriocin, as has also been found in the present investigation. However, the strain *L. lactis* CM1 isolated from Dahi in the

present investigation yields appreciably high bacteriocin activity in a medium with an initial pH of 11.0. This is in contrast to the strain *L. lactis* ATCC 11454, which attained its highest growth and bacteriocin production in a medium with an initial pH ranging from 8 to 9 (Fig. 2). However, the strain failed to grow and thereby to produce bacteriocin in medium with an initial pH of 10.0, implying that the growth of an organism at a high pH is strain-specific. Cheigh et al. reported that the strain *L. lactis* subsp. *lactis* A164 isolated from kimchi produced a nisin-like bacteriocin optimally at pH 6.0 [29]. In TGE medium with an initial pH 6.5, the bacteriocin yield of the strain CM1 was improved when alkali was added to the culture following cessation of growth and bacteriocin production. Nevertheless, the yield was compared to be lower than in cultures started with an initial pH of 11.0 with maltose as the carbon source. We previously reported that *L. lactis* strain W8 could also grow and produce bacteriocin in a medium with an initial pH of 11.0 [30]. The strain W8 was distinguishable from the strain CM1 based on 16S rDNA sequences, biochemical properties, antibacterial activities, and the stability of the bacteriocins. Unlike the strain W8, the strain CM1 utilizes L-arabinose but not gluconate and is VP-negative. However, both strains W8 and CM1 exhibit similar growth patterns on lactose, but the β -galactosidase activity of W8 is almost 10-fold that of CM1 (unpublished observation). No other LAB so far is reported to grow and produce bacteriocin at pH 11.0.

A number of studies have shown that nisin biosynthesis is related to carbon metabolism [31]. Sucrose has been used in the growth media to obtain a maximum stimulating effect on nisin production by *L. lactis* ATCC 11454 [32, 33]. In the present study, we found that the strain 11454 produced the highest level of nisin (3,000 AU/ml) using galactose and trehalose, along with sucrose as carbon source in the medium with a pH of 6.5. The bacteriocin activity of 3,000 AU/ml was also produced by the strain CM1 using maltose as the carbon source, as compared to 2,000, 2,800, and 2,400 AU/ml with galactose, trehalose, and sucrose, respectively, as carbon source. Thus, it appears that nisin biosynthesis and carbon source metabolism of the strain CM1 could be different from that of the strain *L. lactis* ATCC 11454.

The stability of bacteriocin is an important factor for isolation and application for food preservation. The level of bacteriocin produced by the strain CM1 at the end of log phase remained essentially stable, whereas bacteriocin from most producer strains significantly loose their activity due to either protein degradation, adsorption to cell surface, protein aggregation, or complex formation [34, 35].

Nisin Z produced by the strain *L. lactis* CM1 shows greater pH tolerance and thermostability than those reported for other bacteriocins, including nisin of *L. lactis* [36]. Nisin is unstable and inactivated at high pH [37]. The antibacterial spectrum of the bacteriocin, nisin Z, produced by the strain *L. lactis* CM1 is a wide one and is similar to that of nisin. However, *P. acidilactici* F⁺ resistant to nisin of *L. lactis* ATCC 11454 was sensitive to bacteriocin of the strain CM1. *Geobacillus thermoleovorans* and *P. putida* were resistant to the bacteriocin of CM1 but were sensitive to that of the strain W8 [30]. It was reported that both nisin A and nisin Z retained their antibacterial activity after treatment with trypsin [38, 39], whereas nisin Z of CM1 was sensitive to trypsin. The data indicate that the bacteriocin produced by CM1 is similar but not identical to any previously reported nisin. Park et al. [40] also reported a difference in proteolytic sensitivity of nisin Z of *L. lactis* subsp. *lactis* K231 that was also unstable to proteolytic enzymes in contrast to previous observations [38].

The bacteriocin produced by the strain *L. lactis* CM1 did not inhibit the nisin-producing strain *L. lactis* 11454 or vice versa. Because bacteriocin-producing organisms have self-immunity against their own bacteriocin or similar ones produced by others, it is concluded

that bacteriocin of CM1 and ATCC 11454, despite other differences, are recognized as similar for self-immunity. Because the bacteriocin of CM1 has greater pH stability at high temperature and has a wide antibacterial spectrum, the opportunity exists to examine its potentiality for its application in foods as a natural preservative. *Lactococcus lactis* is considered as a GRAS microorganism, and thus, has a potential to be used as a nisin-producing safe starter.

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Nisin, a potent bacteriocin and anti-bacterial peptide, attenuates expression of metastatic genes in colorectal cancer cell lines



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ABSTRACT

Colorectal cancer is the third most common cause of cancer-related death in the world which genetic and environmental agents are responsible for cancer. When cells detach from the tumor and invade surrounding tissues, the tumor is malignant and may form secondary tumors at other locations in a process called metastasis. Probiotics are the largest group of inhabitation bacteria in the colon. Gut microbiota has a central role in prevented the risk colon cancer. Probiotics are beneficial microorganisms, like Lactic acid bacteria and Lactobacilli bacteria which are using in the dairy industry. Probiotics nisin are having the most important category of safe usage. In this study LS180, SW48, HT29 and Caco2 was cultured and treated with different dose of nisin. Cell proliferation was assayed with MTT. The expression of CEA, CEAM6 and MMP2F genes was analyzed with Real-time PCR. Protein expression of CEA was evacuated with ELISA. Our result was shown that the 40–50 IU/mL nisin could suppress proliferation of LS180. Cell proliferation of SW48, HT29, Caco2 cells was decreased in 250–350 IU/mL concentration of nisin. The gene expression of CEA, CEAM6, MMP2F was significantly down-regulated with nisin treatment ($p < 0.001$, $p < 0.01$). Also, after cells treated with nisin, CEA protein expression was down regulated ($p < 0.01$). In conclusion, nisin could suppressed metastatic process via down-regulation of CEA, CEAM6, MMP2F, MMP9F genes. We suggested the new treatment strategies beyond Probiotics, which play a role in the prevention local tumor invasion, metastasis and recurrence.

1. Introduction

Cancer is complex disease that actually comprises more than 100 diseases, with numerous variants. Despite extensive advancements in cancer treatment, many current cancer therapies methods is high toxicity and has side effects for patients [1]. Colorectal cancer occurs when the cells that line the colon or rectum become abnormal and grow out of control. Unfortunately, some colorectal cancers might be present without any signs or symptoms [2]. Colorectal cancer (CRC) has mortality rates vary markedly around the world. CRC is the third most commonly cancer in males and the second in females, with 1.65 million new cases and almost 835,000 deaths in 2015 [3].

Genetic and lifestyle factors are leading causes of colorectal cancer. However genetic factors are not much impressive. Colorectal tumorigenic has increased concentrations of lactate, so we have selected probiotic strains to decrease lactate resistance in an inflamed/tumorigenic environment because probiotics have shown resistance against

acid stress in the initiation of colon cancer cell lines [4]. Mutations in genes that control cell growth and division are responsible for cancer. When cells detach from the tumor and invade surrounding tissues, the tumor is malignant and may form secondary tumors at other locations in a process called metastasis. The best approach in the treatment of colorectal cancer is prevention and combat against the metastases. Spread of this type of cancer remain a serious problem, eventually die of metastatic spread [5]. In recent years, researches focused on application of microbial metabolites, probiotics and toxins in cancer therapy [6,7]. Also, Antimicrobial peptides have been investigated as potential therapeutic drugs and used for preventing bacterial growth in foods. Probiotics are viable microbial species, which are now being studied in various gastrointestinal and non-gastrointestinal disorders. The use of microbials originated with standard dosing, duration of treatment in various diseases to get the consistent results [8]. Nisin A is a lantibiotic bacteriocin produced by *Lactococcus lactis* subsp. *lactis*. It was accepted as a food preservative. Nisin is not active against gram-negative

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bacteria, but liposomes of gram-negative bacteria [9]. and sublethally heat-shocked gram-negative bacteria are inhibited by nisin A [10]. Few studies are available on the anticancer effects of nisin on cancer cells [11–13].

Nisin has been used as an induced preferential apoptosis, cell cycle arrest, and reduces cell proliferation in HN SCC cells, so recently been tested for prevention of growth of cancer cell [14]. It is able to form pores in cells, pore formation induces the release of ions, amino acids, and ATP, so it acts as an anion carrier [15]. Calcium has important role in apoptosis and nisin could changes in intracellular calcium concentration mediated. The mechanisms of metastasis include the intrinsic properties of the tumor cells and the reaction of tissue invasion [14]. Also, it reduced cell proliferation in part the cell cycle arrest mediated by decreased phosphorylation in cdc2 [16].

Human colon cancer associated (carcinoembryonic antigen) CEA that is actually also expressed in during fetal life, absent in the healthy adults. It has been fully characterized and comprised 29 genes. CEA became a favored target antigen for colorectal and other tumors of epithelial origin [17]. It has reported overexpression of CEA & CEACAM6 in colorectal carcinomas cell lines, LS180, Caco-2, SW48, HT29. CEA in the serum after surgery indicated the development of metastasis. CEA has the most important role in the formation of hepatic metastasis and the signaling mechanisms related to colorectal cancer [5].

The identification of neoplastic progression was found to include multiple genetic lesions and overexpression of CEA & CEACAM6 which they could have tumorigenic effects on colonocyte cell lines. The mRNA levels in CEACAM6 is higher than CEA but both overexpression cause loss of cell polarization and inhibition of differentiation and p53 activation [18].

In the current study, we aimed to evaluate the impact of nisin on the metastatic index and alteration in the expression of some main genes; MMP2F, CAM6 and CEA, in the metastatic pathway in colorectal cancer cells.

2. Materials and methods

2.1. LS180 & SW48 & HT29 & Caco-2 cell culture

Cell cultures were used as examination tools for studying cell functions and biotechnology industry. We are using this knowledge for cell counting, cytotoxicity assays, scaling up the number of assays, cell recognition and expression changes caused by treatment [19]. In this experimental work, human colon cancer cell lines, sw48 and HT29 and LS180 were obtained from Pasteur Institute National Cell Bank of Iran in Tehran and plated in T25 flask. The Caco-2 cell line was obtained from Tarbiat Modares university of Iran in Tehran and plated in T75 flask. The cells (HT29,SW48,LS180) were grown in RPMI-1640 medium (Sigma, Germany) which was supplemented with 10% heat-inactivated FBS in 30 min, 568 C (FBS, Gibco, USA), 1% penicillin/streptomycin (provided from Invitrogen, USA)and the Caco-2 were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) high Glucose supplemented with 20% fetal calf serum and 120 U ml⁻¹ penicillin incubated at 37 °C in a humidified atmosphere with 5% CO₂. After they were grown to confluence in 75 cm² tissue culture flasks. We harvested and washed twice with PBS, so cells were detached and transferred to new cell culture. Trypsin-EDTA was used for cells detachment after confluence. Mono layers were prepared on placed in 96-well tissue culture plates. Cells were seeded at a concentration of 5 × 10³ cells per well to obtain confluence. Those cell lines were treated by Nisin and were studied with relevant techniques. (MTT, Trypan blue) The cells were examined by adding 20,40, 50,150, 200, 250, 300, 350, 400, 450 IU/mL of probiotic Nisin after 24 h incubation at 37 °C. Seven strains were tested at the same time in duplicate in Trypan blue were measurement of alive cell viability. The cells were prepared for obtain of optimum dose of Nisin in colon cancer treatment.

2.2. Determination of optimum dose of Nisin in colon cancer treatment

2.2.1. Trypan blue exclusion assay

The Trypan blue test is a practical method for selective stains dead cells. The 96-well plate was used to measure the inhibitory effect of Nisin on colon cancer cell lines. A total of cells (5 × 10³ cells/well) were seeded in each well containing RPMI medium (DMEM high Glucose for Caco-2), 10% FBS, and 1% penicillin/streptomycin. After 24 and 48 h, each duplicate well was treated with a range of nisin concentration (50–1000 IU/mL). The main advantage of this assay is that you don't need to resolubilize and the cells metabolize. After overnight I have used the trypan blue staining method along with a microscope. The Viability is just the ratio of live cells divided by total number of cells. Caco-2, LS180, SW48 and HT29 cells were grown in 96-well plates after incubated cells were treated.

2.2.2. MTT assay

Cell viability was evaluated biochemically with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. This assay relies on the ability of viable cells to metabolically. The assay was carried out under the same experimental conditions as Trypan blue. 5 × 10³ cells was seeded in a 96-well plate in 100 µl of complete medium, after about 24 and 48 h, cells in each well were treated, by Nisin. Then, 10 µl MTT (5 mg/mL) were added to each well, including controls, and the culture plate returned to the cell culture incubator for 4 h. The MTT molecule needs to enter a cell and functioning mitochondria to be converted so it is a metabolic assay and get converted to Formazan using NADPH. After this time the medium was carefully removed and 100 µl DMSO (resolubilize the formazan and to kill cells) were added to dissolve the intracellular punctuate purple precipitate that is proportional to the number of viable living cells. The absorbance was read at 570 nm using an ELISA reader (VersaMax ELISA Microplate Reader, CA, USA). Each experiment was duplicate.

2.3. Analysis of metastasis genes expression by quantitative RT-PCR analysis

2.3.1. RNA extraction

Total RNA was isolated from colon cancer cell lines using Sinagen RNA extraction kit (Sigma, USA) according to the manufacturer's protocol. The concentration and purity of RNA were determined by spectrophotometry (NanoDrop, Wilmington, DE). After treated the cells with different concentration of nisin, cell washed with PBS and added 1 mL RNAX-Plus (Cinagen, Iran) After 5min, cold choloroform was added and centrifuge at 12000 rpm at 4°C 15min. Aqueous phase contained total RNA. About 200 µl Aqueous phase mix with equal volume of isopropanol and centrifuge the mixture at 12000 rpm at 4°C 15min. In the following discard the supernatant and add 75% Ethanol then centrifuge at 4°C for 8 min at 7500 rpm. Dissolve pellet in 30 µl of DEPC treated water. The obtained RNA is ready for use in, cDNA synthesis, RT-PCR and etc. The concentration of RNA was measured at 260/280 nm absorbance ratios of 1.8–2.0, which indicated a pure RNA sample.

2.3.2. cDNA synthesis

To assess the effects of nisin on the expression of metastases markers in colon cancer cell lines, 1 µg of RNA was used to be converted into cDNA using the First Strand cDNA Synthesis Superscript kit (Invitrogen) according to the manufacturer's protocol. RT-PCR experiments were carried out with the following synthesized GapDH-F primer and GapDH-R primer as an internal control: 5'-AGAAGGCTGGGGCTAT TTG-3' and 5'-AGGGGCCATCCACAGTCTTC-3' reactions were optimized for the 40 cycles to ensure product intensity within the logarithmic phase of amplification.

2.3.3. Real-time PCR

After RNA extraction, cDNA was synthesized using Primescript RT Reagent. Real time PCR was carried out using Green PCR Super Mix (BioRad, CA USA), and conducted following the Quantitative real-time PCR procedure defined by constructor (Rotor gene Q, Germany). Relative mRNA expression was quantified using the $\Delta\Delta Ct$ method and the GapDH was used as an internal control.

2.4. Metastasis analysis using kit CEA & CEAM6 for cell ELISA

Abcam's Carcinoembryonic Antigen (CEA & CEAM6) Human ELISA (Enzyme-Linked Immunosorbent Assay) kit is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of Human CEA & CEM6 in serum, plasma, and cell culture supernatants. Many studies have confirmed carcinoembryonic antigen (CEA & CEAM6) levels and metastasis. This assay employs an antibody specific for Human CEA & CEAM6 & MMFP2 coated on a 96-well plate. This protein level had shown by changing the color from blue to yellow, and the intensity of the color were measured at 450 nm. Calculate the mean absorbance for each cell lines set of duplicate standards, controls and samples.

2.5. Statistical analysis

Results are expressed as mean SD of three independent experiments. Differences between groups were compared using Student's *t*-test; $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Evaluation of cell death via Trypan blue

To compare the cell cytotoxicity effect of nisin on HepG2 cells Trypan blue assays was used (Fig. 1). Cell viability was determined over 2 days. After cells (ls180, SW48, HT-29 and Caco-2) were cultured in 96 well-plates, the viability was decreased in a dose-dependent manner by using the Trypan blue assay. Interestingly, the cell viability was considerably decreased about 50% for 24 h after 80–400 IU/mL nisin on LS180 cancer cells in the Trypan blue assay (Fig. 1A.). But, in SW48, HT-29 and Caco-2 cells the cell viability was decreased about 50% on 350–800 IU/mL nisin. Indeed, compare to LS180 higher concentration of nisin needed for suppressing proliferation of SW48, HT-29 and Caco-2 cells.

3.2. Effect of nisin on colon cancer cell lines' viability under nisin treatment

The MTT method was used for evolution the cytotoxicity effect of nisin on colon cancer cell lines. Colon cancer cells (5×10^3) were treated with different concentration of nisin for 24 h in duplicate. Significantly, LS180 Cell death was started in 50 IU/mL of nisin (Fig. 2A.). Also, in SW48, HT29, Caco-2 cell lines, the survival was significantly decreased in 350–800 IU/mL of nisin incomparation to control group (untrated cells) but there was not cell death on lower than 200 IU/mL nisin in treated cells in compare to control group (Fig. 2A–D).

3.3. Investigation of metastatic genes expression (CEA, CEAM6, MMP2F)

To assess the impact of nisin on metastatic genes expression, 5×10^5 cells were seeded in 6-well plates and treated with nisin. The mRNA expression of CEA, CEAM6, MMP2F genes was determined by Real-time PCR. As shown in Fig. 3, considerably the ratio expression of metastatic genes were considerably suppress after treated of colon cancer cell lines with nisin compared to the untreated cells ($p < 0.05$). In presence of nisin, the ratio of CEA expression was decreased in colon cancer cell lines (LS180, sw48, HT-29 and Caco-2) about 3 fold (Fig. 3A–D.). Also, against nisin, cancer cell lines-nisin group was

shown low expression of CEAM6 compared control group (untreated group) (Fig. 3.). Moreover, the ratio of MMP-F2 expression was significantly reduced after exposing to nisin treatment ($p < 0.001$) (Fig. 3A and D). Treating cells with 250, 500 and 700 IU/mL of nisin can suppress metastatic gene in colon cancer cell lines.

3.4. Decrease CEA protein levels in colon cancer cell lines via nisin

In this section, measured suppress CEA protein expression in culture medium of colon cancer cell lines that treatment by different concentration of nisin by ELISA kit. To calculate fold changes in ELISA, the obtained value of treated colon cancer cell lines cells was divided by the value obtained from the control samples (untreated cells). The ELISA again verified the real-time RT-PCR results. In all samples (SW4, LS-180, Caco2, HT-29), CEA protein was decreased compared to the controls (untreated samples) (Fig. 4). In SW48, HT29, Caco-2 cell lines, CEA protein level was reduced in 250 and 300 IU/mL nisin. The same of CEA gene expression in LS180 cell line, the 40 and 50 IU/mL concentration of nisin caused suppressing protein expression. There was a positive correlation between CEA concentration and fold changes in real-time.

4. Discussion

In this paper we have used human colon cancer cell lines, HT29, Caco-2, LS180, SW48 to study the effect of probiotic Nisin for poor prognosis of cancer. Metastasis-associated with colon cancer is an evolutionarily [20]. According to the Knychalski B studied was a positive link between CEA in patients with colon cancer which patients with colorectal cancer had higher concentrations of CEA. They show all concentrations of nisin tested significantly reduced mean tumor volumes [21]. Following previous studies, in Tehran University in 2015 was shown that in the prevention of metastasis in HT29 cells with decreased expression of MMP2, MMP9 and increased expression of their inhibitors [22]. Our findings showed nisin derived from *Lactococcus lactis* which is useful anti metastatic therapeutic effect on colon cancer cell lines. Thus nisin treatment extended, it has abilities for inducing apoptosis even at the highest tested dose of 800 IU/mL (Kamarajan P et al., 2015). Also, on 2018 report, nisin induced selective toxicity in melanoma cells compared to non-malignant keratinocytes. Furthermore, nisin Z increase reactive oxygen species generation and cause apoptosis and can decrease the invasion and proliferation of melanoma cells demonstrating its potential use against metastasis [23]. In our study, 250 IU/mL of nisin significantly decreased cell viability of LS180 and the concentration of 450–500 IU/mL is cytotoxic effect HT29, Caco-2, and SW48. Many studies have shown an association between serum carcinoembryonic antigen (CEA) and CEAM6 levels and metastasis. So, MMP2F expression correlates with metastatic abilities and poor prognosis (Y.-S.Hsieh et al. and O. Bajenova _ P. Thomas). It is confirmed that metabolites nisin and lactobacillus species including LAB and *L. rhamnosus* motivated the apoptosis against cancer cells. This is happened through the interaction of probiotics and their via secretion including bacteriocins and extracellular [24]. Our Data about CEA Elisa was indicated that nisin decreased the CEA protein expression on condition medium of colon cancer cell lines (HT29, Caco-2, LS180, SW48).

In University of Michigan studies has shown probiotics treatment decreases phosphorylation of checkpoint marker like cdc2 in HNSCC cells. So, it has compared with β -actin which, it was stable total protein of cdc2 [16,25]. Annarita Farina et al. reported CEA and CEAM6 levels in the malignant tumor have increased. CEA and CEAM6 have expressed by epithelial cells and secreted in to adjoining fluids, that correlate with metastases and as a biomarker for pancreaticobiliary barriers. ELISA test has identified CEAm6 as a known biomarker and mechanisms of transport [26]. The same of Annarita Farina et al.; in this study CEA Elisa test was used for metastatic biomarker but CEAM6

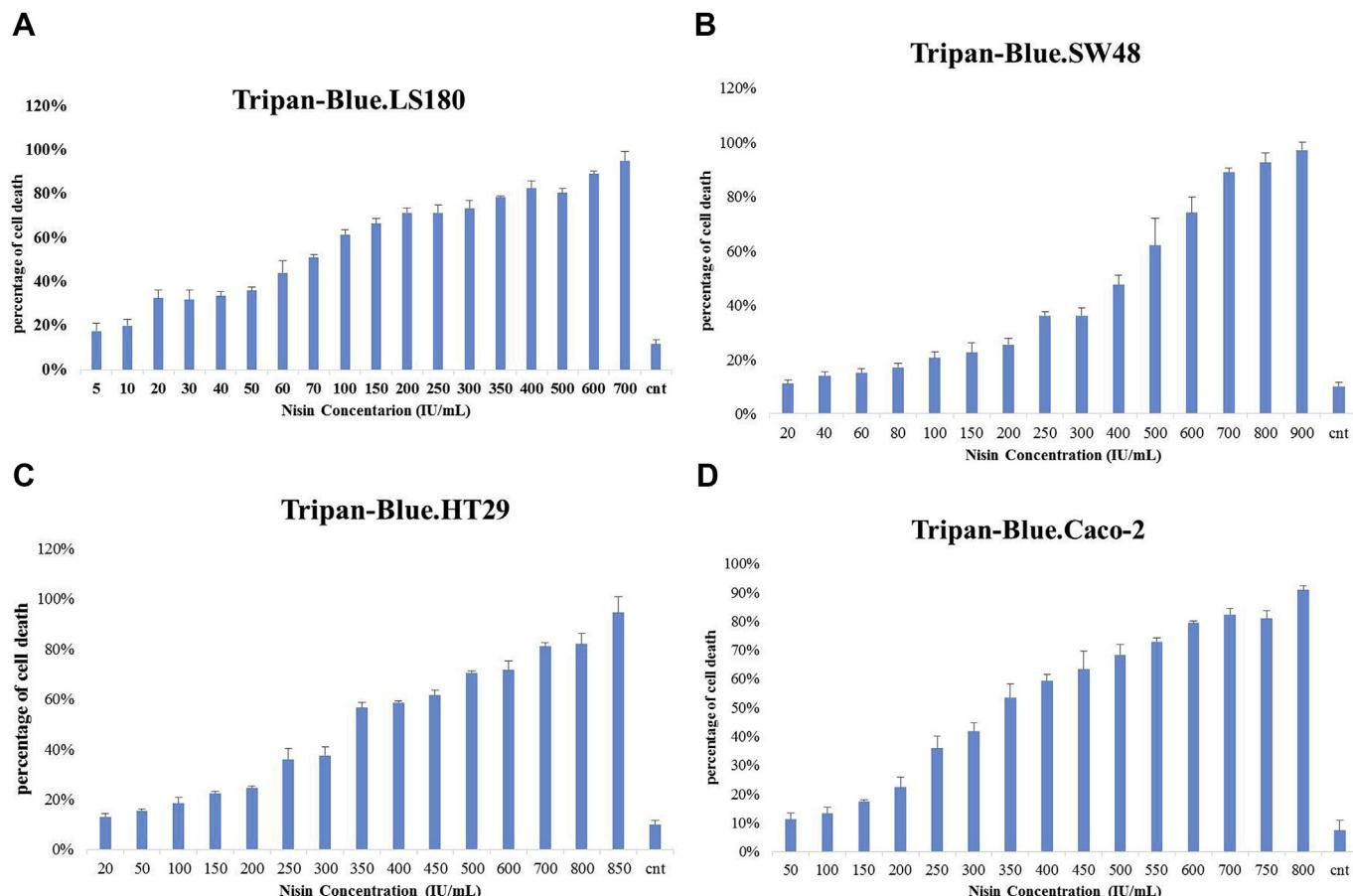


Fig. 1. Cytotoxicity results for colon cancer cells following nisin exposure determined by Trypan Blue. A. LS180; B. SW-48; C. HT-29; D. Caco-2. Ls180 were inhibited by 20, 40, 50 IU/mL doses of Nisin, sw48, Caco-2 and HT29 were inhibited by 200, 250, 300 IU/mL des of Nisin. (mean \pm SD of three independent experiments; ** $p < 0.01$ and *** $p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and MMP-2 levels was assayed in gene expression level by Real-time PCR. In analyzing by Zhihua Yao, MMP-2 and MMP-9 expression associated with lymph node metastasis. In addition, MMP-2 and MMP-9 have had important role in collagen and tumor histological grade. MMPs are inducer tumor cells metastasis through ECM and antiepathic activity. MMPs have important role in cancer development and regulating signal pathways [27]. As shown in Fig. 3, cancer cell lines-nisin group was shown to have low expression of expression of CEAM6 compared control group (untreated group). Moreover, the ratio of MMP-F2 expression was significantly reduced after exposing to nisin treatment ($p < 0.001$). Treating cells with 250, 500 and 700 IU/mL of nisin can suppress metastatic gene in colon cancer cell lines.

According to Sam Maher et al., investigated the first the cytotoxicities comparing of prokaryotic and eukaryotic on gut epithelial cells. LAB produced from prokaryotic, was one of the important antimicrobials in dealing of numerous infections. HT29 and Caco-2 cells were incubated with Cytotoxic Nisin for 24 h which this study shown Nisin used such as infections (example, intestinal, vaginal, and infections of the skin) [28]. In this study we suggested the new treatment strategies beyond Probiotics, which play a role in the prevention local tumor invasion, metastasis and recurrence. LS180, SW48, HT29, Caco2 was cultured and treated with different dose of nisin. Cell proliferation was assayed with MTT. The expression of CEA, CEAM6, MMP2F, MMP9F genes was analyzed with Real-time PCR. Protein expression of CEA was evacuated with ELISA. Our result was shown that the 40–50IU/mL nisin could suppressed proliferation of LS180. Cell proliferation of range SW48, HT29, Caco2 was decreased in 250–350 IU/mL concentration of nisin. The gene expression of CEA, CEAM6,

MMP2F, MMP9F was significantly down-regulated with nisin treatment ($p < 0.001$, $p < 0.01$). Also, after cells treated with nisin, CEA protein expression was down regulated ($p < 0.01$). In conclusion, nisin could suppressed metastatic process via down-regulation of CEA, CEAM6, MMP2F, MMP9F genes.

5. Conclusion

MMPs and CEA are the important molecular biomarkers for detection of colon cancer metastasis. This study investigated the anti-metastatic effect of nisin on cultured HT-29, Caco2, LS180 and SW48 colon cancer. We have suggested that the first stages of metastasis can be blocked by anti-metastatic effects of nisin. Treatment with probiotic nisin reduced the expression of these MMPs and CEA. The total expression was lower in HT-29, Caco2, LS180 and SW48 cells after nisin treatment, compared to control (untreatment cancer cells). However, to clarify the anti-metastatic mechanisms of nisin, the additional investigations are required.

Conflicts of interest

All authors declare that they have no conflict of interest.

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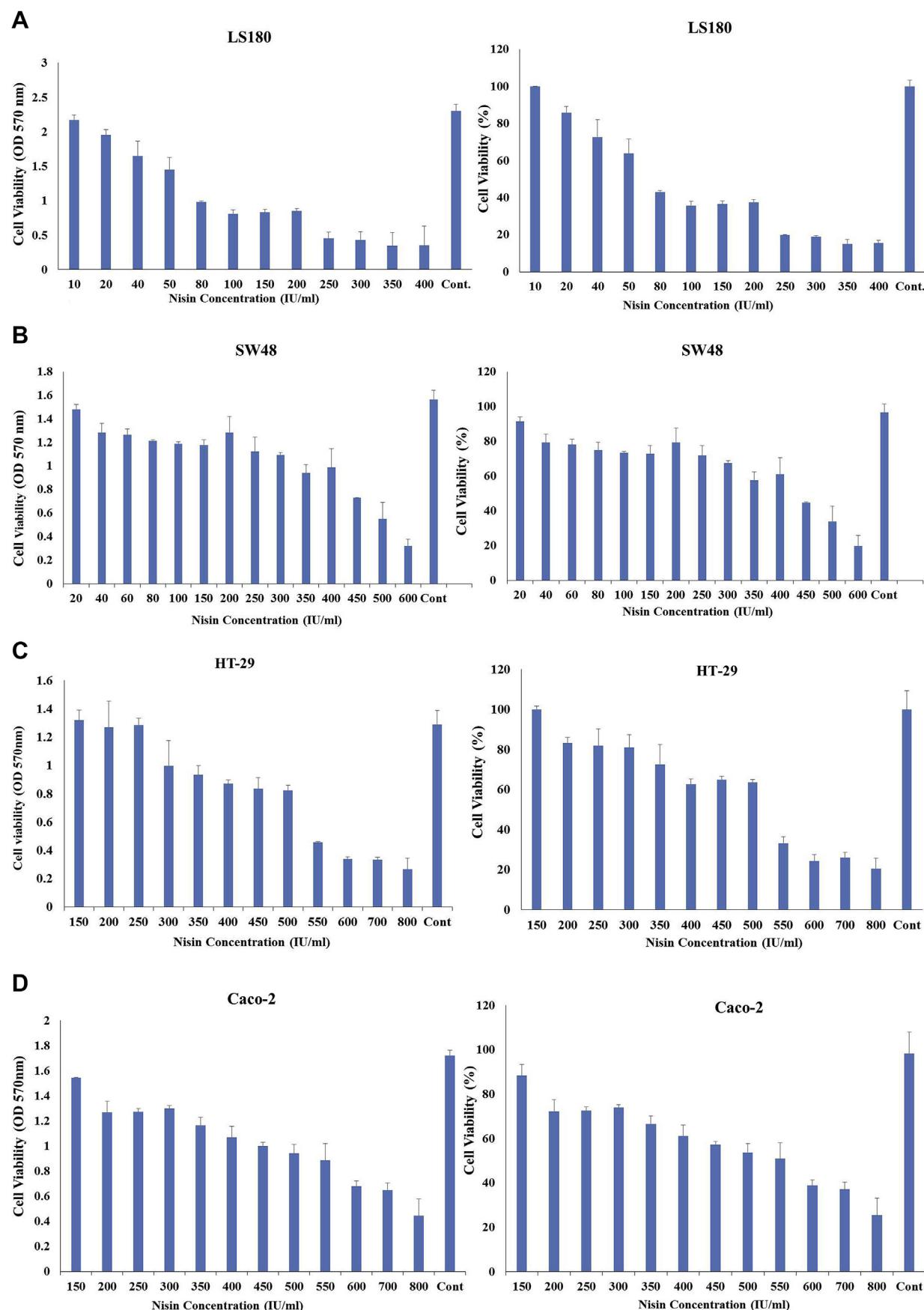


Fig. 2. Cell viability of colon cancer cells following nisin exposure determined by MTT assay. The Cells were cultured in 96-well Plates to analyze their viability by the MTT Assay. A. LS180; B. SW-48; C. HT-29; D. Caco-2. The results established that nisin can decrease proliferation in compared to control group (cells without treated). (mean \pm SD of three independent experiments; ** p < 0.01 and *** p < 0.001).

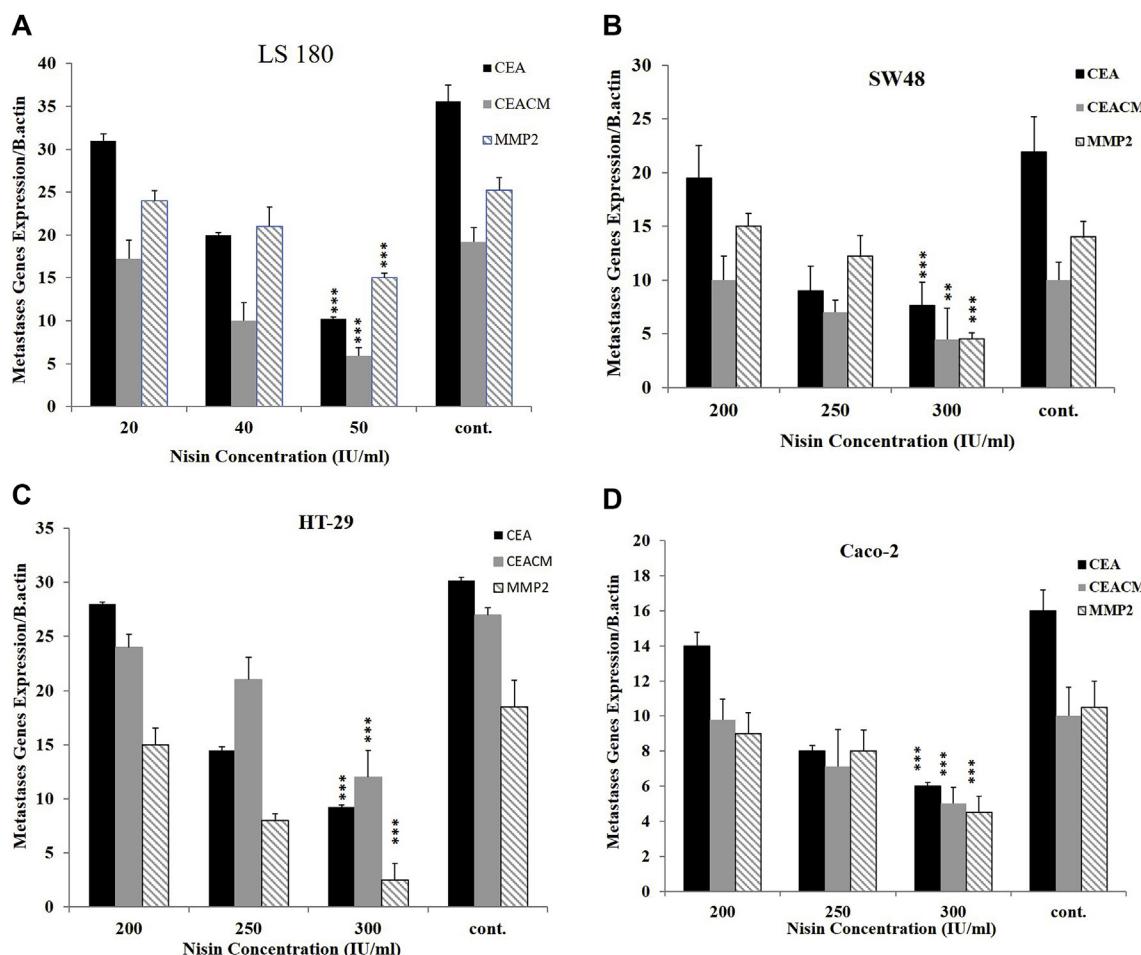


Fig. 3. Relative mRNA expression of metastatic gene colon cancer cells following nisin exposure; A. LS180; B. SW-48; C. HT-29; D. Caco-2. Quantitative assessment of the expression levels of CEA, CAM6, MMP2 was performed with real-time PCR. Interestingly, in treated cells expression level of the CEA, CAM6, MMP2 genes was obviously lower than untreated cells. (mean \pm SD; * p < 0.5, ** p < 0.01, and *** p < 0.001; number of replicates, 3).

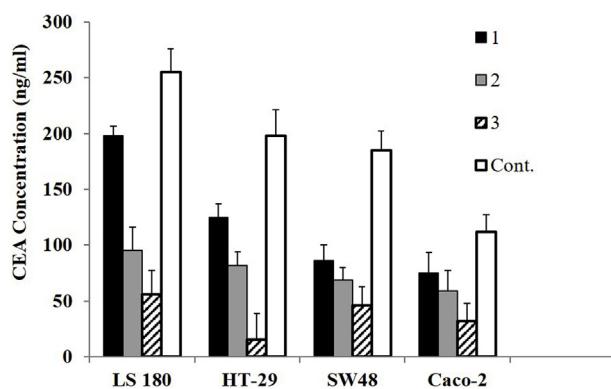


Fig. 4. Elevated nisin suppress CEA protein in colon cancer cell lines. Colon cancer cell lines (Ls180, sw48, Caco-2 and HT29) were treated with 20–400 IU/mL nisin at indicated time points. CEA secretion was measured by enzyme-linked immunoabsorbent assay (ELISA). Data are presented as mean \pm SD from two individual donors. The lowest level of the CEA was observed in colon cancer cell lines that treated with nisin, while the control group showed the highest levels of the CEA (mean \pm SD of three independent experiments; ** p < 0.01 and *** p < 0.001).

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

For this type of study, formal consent is not required.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.micpath.2018.07.006>.

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