



Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage: www.elsevier.com/locate/apjtd



Document heading doi:10.1016/S2222-1808(12)60047-4

## Detection of intestinal colonization of probiotic *Lactobacillus rhamnosus* by stool culture in modified selective media

Manisha DebMandal<sup>1,2</sup>, Shyamapada Mandal<sup>1\*</sup>, Nishith Kumar Pal<sup>1,3</sup><sup>1</sup> Department of Bacteriology and Serology, Calcutta School of Tropical Medicine, C. R. Avenue, Kolkata-700073, India<sup>2</sup> Department of Physiology and Biophysics, KPC Medical College and Hospital, Jadavpur, Kolkata-700032, India<sup>3</sup> Department of Microbiology, Institute of Postgraduate Medical Education and Research, Kolkata-700020, India

## ARTICLE INFO

## Article history:

Received 15 March 2012

Received in revised form 27 April 2012

Accepted 28 May 2012

Available online 28 June 2012

## Keywords:

*Lactobacillus rhamnosus* GG

Selective media

Stool culture

pH

Antibiotic

## ABSTRACT

**Objective:** To develop an approach for making a selective and differential media specific for *Lactobacillus rhamnosus* Goldin and Gorbach (LGG) in order to distinguish it from other bacterial flora in fecal samples. **Methods:** The current media referred as LGGM has been prepared by using MRS media ingredients (replacing dextrose with 2% sorbitol), and incorporation of nalidixic acid (40 µg/mL), bromocresol purple (0.002 %); the pH of the media was adjusted to 4.5. **Results:** The LGGM showed a greater colony forming units compared to MRS media. The growth of pure LGG strain was significantly greater in LGGM, and the recovery rate of LGG released from fecal samples (after oral feeding) was significantly greater ( $P < 0.001$ ). **Conclusion:** Thus LGGM could be used as a potential media for the isolation and identification of LGG strain.

### 1. Introduction

Probiotics have been defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host [1]. The potential and established effects of probiotic bacteria include aid in lactose digestion, resistance to enteric pathogens, anti-colon cancer effect, immune system modulation, allergy, blood lipids, heart disease, antihypertensive effect, urinary tract infection, hepatic encephalopathy, prevention of dental caries, respiratory infections and inflammatory bowel disease [1, 2] *Lactobacillus rhamnosus* Goldin and Gorbach (LGG) is the most extensively studied probiotic in children and adults [3, 4]. It has been used in the prevention and management of diarrheal diseases. Grandy *et al* [5] have shown that LGG is effective in preventing rotaviral diarrhea in children. There is evidence that LGG, given orally at doses  $> 10^9$  cfu/day, colonizes the intestine, and reduces diarrhea, and it has been concluded that the minimal dose required to have a positive effect was  $5 \times 10^9$  cfu [3]. LGG reduces the incidence of antibiotic-

associated diarrhea in children treated with oral antibiotics for common childhood infections [1].

The use of probiotics in India dates back to thousands of years with the use of curd (yoghurt) as a beneficial food supplement in almost each and every household. In light of the current public and scientific interest in probiotics and the newly revealed possibilities for scientific exploration, the effectiveness of the LGG strain on a diarrheal endemic region, Bhatpara (suburb Kolkata) of the West Bengal state, India, was evaluated. The LGG strain survives gastric acidity, bile, and has a very high capacity of adhesion to the villi of the small intestine, and in this way colonizes the human digestive system on oral administration and with regard to persistence, LGG has to be recovered from feces, after ingestion, in a bacteriological media [6–8]. However, Lactobacilli adapted prepared media is presented with the growth of other intestinal bacterial flora causing problems in the detection of LGG. Thus the present study was carried out to modify selective media for the detection and quantification of LGG from fecal samples in order to ensure the colonization of and persistence in human intestine after oral administration of LGG.

### 2. Materials and methods

\*Corresponding author: Dr. Shyamapada Mandal, Department of Zoology, Gurudas College, Narkeldanga, Kolkata-700 054, India.  
E-mail: samtropmed@gmail.com

### 2.1. Bacterial strains

The original LGG (~10<sup>9</sup> live bacteria, Culturelle; Amerifit Brands, Connecticut, USA) strain was kindly provided by Dr. S. Misra, UIC College of Medicine at Peoria, USA to Dr. N.K. Pal, one of the authors of the paper. Clinical isolates of *Escherichia coli* (*E. coli*), *Klebsiella aerogenes* (*K. aerogenes*), *Salmonella enterica serovar Typhi* (*S. typhi*), *Shigella sonnei* (*S. sonnei*), *Proteus vulgaris* (*P. vulgaris*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), *Vibrio cholerae* (*V. cholerae*) were used as tester strains.

### 2.2. Media and chemicals

In this study, MRS medium (Acumedia Manufacturers, Maryland, USA) was used. All the chemicals, media and antibiotics were purchased from Himedia, Mumbai, India, unless otherwise specified.

### 2.3. Stool samples

Stool samples utilized in the study were obtained from Bhatpara State General Hospital (BSGH), West Bengal, India; kindly provided by Dr. Soumava Datta, Superintendent, BSGH, from children <36 months old with diarrhea, who were treated with LGG oral doses during their hospitalization. The stool samples were brought to the Calcutta School of Tropical Medicine, India, within 2 h for further study.

The collected samples were diluted in sterile PBS (1g in 10 mL), and then homogenized in a vortex mixer and suitable dilutions were made for counting the colonies [9] after 48 h growth under anaerobiosis at 35 °C using both LGGM and MRS agar media. A loopful suspension of the fecal samples were streaked onto media plates and incubated in 10 % CO<sub>2</sub> and 10 % hydrogen at 35 °C for 48–72 h. Well isolated colonies with typical characteristics were selected and transferred to MRS broth.

### 2.4. Preparation of bacterial inocula

The LGG strain was pre-grown in MRS broth (pH 6.5) and incubated at 35 °C for 24 h. The broth culture was diluted serially in fresh MRS broth, and inoculum was adjusted to approximately 5 × 10<sup>5</sup> cfu/mL (5.698 log<sub>10</sub> cfu/mL) [9].

### 2.5. Biochemical characterization

The cultures were characterized for their morphological, cultural, physiological and biochemical characteristics. The used tests were: Gram reaction; production of catalase in phosphate buffer at pH 7.0, cytochrome oxidase and hydrogen peroxide; growth at 15 °C and 45 °C in 1 week; acid production from carbohydrates (1 % w/v) – L-arabinose, cellobiose, D-fructose, D-galactose, lactose, melezitose, melebiose, mannitol, D-mannose, salicin, sorbitol, trehalose and D-xylose in MRS broth devoid of glucose and beef extract with chlorophenol red as indicator; production of acid and gas from 1 % glucose (MRS broth without beef

extract); methyl red and Voges–Proskauer test; production of ammonia from arginine; nitrate reduction in nitrate broth; indole production in tryptone broth and growth on acetate agar.

### 2.6. Effect of pH on growth of LGG

The pH of the MRS media was adjusted to 3, 4, 4.5, 5.0, 5.5, 6.0, 6.5, 7 and 7.5 using 2 % HCl, in order to assess the growth of LGG strain, with 5 × 10<sup>5</sup> cfu/mL initial inocula, following 48 h incubation at 35 °C.

### 2.7. Antibiotic susceptibility test by disc diffusion method

Antibiotic susceptibility of the LGG strain was tested by disc diffusion method with an inoculum of approximately 10<sup>8</sup> cfu spread on MRS agar plate. The antibiotic discs used in the study were polymyxin B (Pb, 300 units), nalidixic acid (Nx, 30 µg), cefotaxime (Cm, 30 µg), levofloxacin (Lf, 5 µg), ampicillin (Am, 10 µg), cotrimoxazole (Co, 25 µg), gentamicin (Gm, 10 µg), furazolidone (Fz, 100 µg), ciprofloxacin (Cp, 5 µg) and ceftriaxone (Cn, 30 µg), norfloxacin (Nf, 10 µg). The discs were placed on the agar plates, and incubated at 35 °C for 48 h. The zone diameter of inhibition (ZDI) around each antibiotic disc was recorded and the strains were accordingly interpreted as sensitive, intermediately susceptible, and resistant.

### 2.8. Effect of different concentration of antibiotics

Effects of 11 different concentrations (ranging from 5 to 50 µg/mL) of Pb, Nx, Cm, Lf, Am, Co, Gm, Fz, Cp, Cn, Nf were studied against the LGG strain. Overnight grown bacterial suspension was diluted to approximately 5 × 10<sup>5</sup> cfu/mL in fresh MRS (pH 4.5) broth with appropriate antibiotic concentration, and incubated at 35 °C. The samples after 24 h incubation were diluted and plated on MRS agar (pH 4.5) to determine viable cell counts after 48h at 35 °C.

### 2.9. Modification of selective media

The selective medium designated LGGM (*L. rhamnosus* GG medium) was modified based on the resistance of the LGG to Nx (40 µg/mL), ability to show maximum growth of 9.2 log<sub>10</sub> cfu/mL at pH 4.5, and its ability to produce acid from sorbitol. The formulation of medium was peptone 1%, yeast extract 0.5%, beef extract 1%, sodium acetate 0.5%, polysorbate 80 0.1%, disodium phosphate 0.2 %, ammonium citrate 0.2%, magnesium sulphate 0.01%, manganese sulphate 0.005%, D-sorbitol (2%), bromocresol purple (0.002 %) nalidixic acid (40 µg/mL), agar (2%). The LGGM was purple colored after autoclaving and solidification. The LGGM was tested by streaking onto the media plates a loopful suspension of a cocktail of test bacteria mentioned above as well as the individual strains separately. The plates were incubated under anaerobiosis at 35 °C for 48–72h.

### 2.10. Statistical analysis

LGGM was evaluated and compared with MRS media using  $\chi^2$ -test with both original strain of LGG and fecal LGG.

**3. Results**

The LGG strain, which grew in LGGM and MRS medium, was incapable of growing in bacteriological media like nutrient agar, MacConkey agar, Salmonella–Shigella agar, desoxycholate agar, thiosulphate citrate bile sucrose agar. The *E. coli*, *K. aerogenes*, *S. enterica Typhi*, *Sh. sonnei*, *Pr. vulgaris*, *Ps. aeruginosa*, *St. aureus* and *V. cholerae* strains failed to form colonies on LGGM agar.

On LGGM, the LGG strain produced yellow colonies surrounded by a yellow halo after 48 h at 35 °C. In MRS agar the LGG colonies were convex, round, smooth, opaque, and white, which turned pale, rough upon prolonged incubation beyond 7 days. The LGG (gram positive non–sporulating, non–motile bacilli) in anaerobiosis appeared stout with a length of 10  $\mu$ m or more and width 0.2 – 0.3  $\mu$ m; the cells were often arranged in chains with the long axis slightly curved. The bacilli, however, in aerobiosis showed shorter (length about 7 to 8  $\mu$ m) and thinner (width 0.2  $\mu$ m) cells having less intense gram staining.

The biochemical characteristics of the lactobacillus

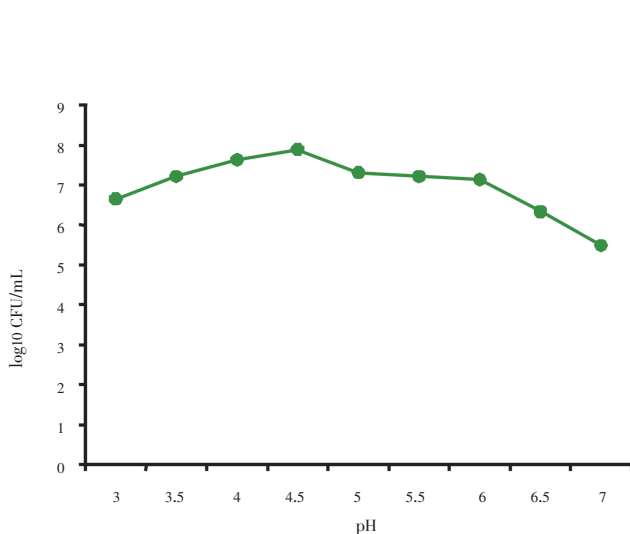
species is shown in Table 1. The LGG strain was capable of fermenting several sugars including mannitol, salicin, sorbitol, cellobiose, mannose, melezitose, xylose, melebiose; however no acid and gas production occurred from lactose and glucose. Table 2 depicts the growth patterns of the test bacterial strains including LGG in MRS agar media with different pH values (3–7.5). The LGG strain grew at the pH range 3 to 7; at pH 6.5, LGG along with the *E. coli*, *Pr. vulgaris* and *St. aureus* formed colonies, while the remaining bacterial strains did not grow.

**Table 1**  
Biochemical characteristics of *L. rhamnosus* GG.

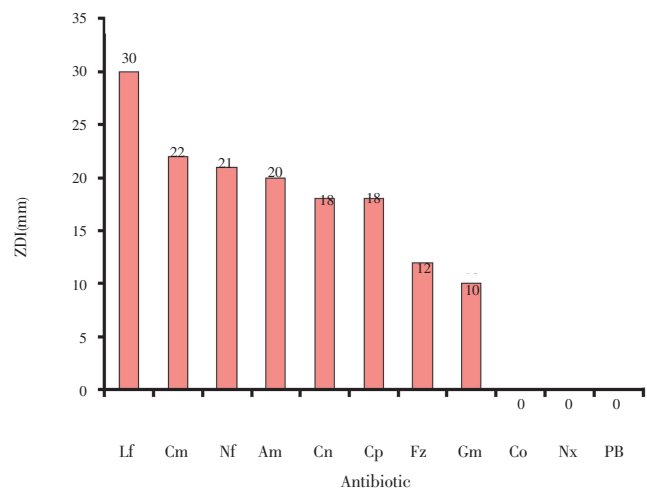
Test	Result	Test	Result
Growth at 15 °C	–	Mannose	+
Growth at 45 °C	–	Lactose	–
Growth at 35 °C	+	Acid and gas from glucose	–
Catalase	–	Motility	–
NH <sub>3</sub> from arginine	–	Triple sugar iron	Alkaline/acid
Mannitol	+	H <sub>2</sub> S production	–
Salicin	+	Melezitose	+
Sorbitol	+	Xylose	+
Cellobiose	+	Melebiose	+

**Table 2**  
The effect of pH on bacterial growth in MRS agar

pH	Bacteria									
	<i>L. rham-nosus</i> GG	<i>E. coli</i>	<i>K. aerogenes</i>	<i>S. typhi</i>	<i>S. sonnei</i>	<i>P. vulgaris</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>V. cholerae</i>	
3	+	–	–	–	–	–	–	–	–	
3.5	+	–	–	–	–	–	–	–	–	
4	+	–	–	–	–	–	–	–	–	
4.5	+	–	–	–	–	–	–	–	–	
5	+	–	–	–	–	–	–	–	–	
5.5	+	–	–	–	–	–	–	–	–	
6	+	–	–	–	–	–	–	+	–	
6.5	+	+	–	–	–	+	–	+	–	
7	+	+	+	–	–	+	–	+	–	
7.5	–	+	+	–	–	+	–	+	–	

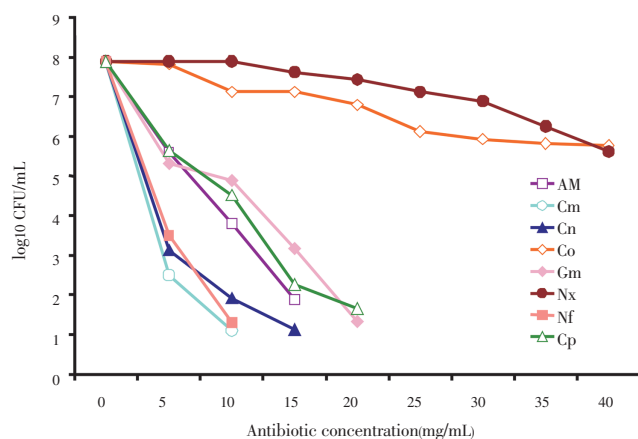


**Figure 1.** Influence of pH on colony forming unit (cfu) in MRS broth for LGG.

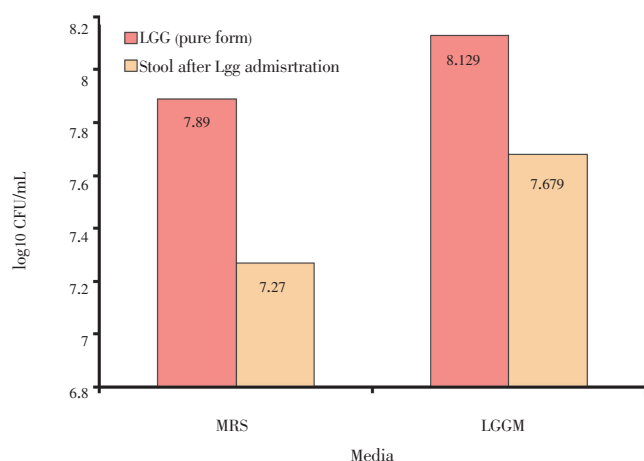


**Figure 2.** Antibiotic susceptibility test results in terms of ZDI (mm) for LGG. PB; Polymixin B, Nx; Nalidixic acid, Cm; Cefotaxime, Lf; Levofloxacin, Am; Ampicillin, Co; Cotrimoxazole, Gm; Gentamicin, Fz; Furazolidone, Cp; Ciprofloxacin, Cn; Ceftriaxone, Nf; Norfloxacin.

Figure 1 shows the colony forming units of LGG on the MRS agar plates (pH 3 to 7); maximum growth in terms of cfu ( $7.89 \log_{10}$  cfu/mL, i.e.,  $\sim 7.76 \times 10^7$  cfu/mL) was noticed at pH 4.5. The ZDI for LGG strain in MRS agar after 48 h incubation at 35 °C for Lf, Cm, Nf, Am, Cn, Cp, Fz, Gm, ranged 10–30 mm; for Co, Nx, Pb no ZDI was obtained (Figure 2). The effect of various concentrations (0–40  $\mu$ g/mL) of different antibiotics on  $5 \times 10^5$  cfu/mL ( $5.698 \log_{10}$  cfu/mL) of LGG, which was used as the initial inocula in MRS broth, has been represented in Figure 3. The inhibitory effect on bacterial growth was observed in presence of Cm and Nf, and beyond concentration 10  $\mu$ g/mL for both the antibiotics no growth was found. A growth of 1.88 and 1.12  $\log_{10}$  cfu/mL was obtained, respectively in presence of Am and Cn (15  $\mu$ g/mL each). The Gm and Cp, with 20  $\mu$ g/mL allowed a growth of 1.322 and 1.66  $\log_{10}$  cfu/mL, respectively; the growth was fully inhibited above concentration 20  $\mu$ g/mL. Growth was up to 5.62 and 5.77  $\log_{10}$  cfu/mL at 40  $\mu$ g/mL Co and Nx, respectively.



**Figure 3.** Effect of antibiotic on growth of LGG in MRS broth. PB; Polymixin B, Nx; Nalidixic acid, Cm; Cefotaxime, Lf; Levofloxacin, Am; Ampicillin, Co; Cotrimoxazole, Gm; Gentamicin, Fz; Furazolidone, Cp; Ciprofloxacin, Cn; Ceftriaxone, Nf; Norfloxacin.



**Figure 4.** Growth of LGG strain (original and after oral administration in stool culture) in MRS and LGGM

The LGGM was evaluated for the recovery of LGG in stool samples from diarrheal patients (as mentioned above); approximately  $7.679 \log_{10}$  cfu/mL ( $4.8 \times 10^7$  cfu/mL) of the LGG strain has been recovered in fecal discharge, after oral administration of  $\sim 10 \times 10^8$  live bacteria; the MRS presented

with a growth of  $7.27 \log_{10}$  cfu/mL ( $1.9 \times 10^7$  cfu/mL), as shown in Figure 4. The original LGG strain was enumerated both in LGGM and MRS media, which gave 7.89 and  $8.129 \log_{10}$  cfu/mL, respectively.

#### 4. Discussion

For the detection of *Lactobacilli*, different types of media are used, such as tomato–juice medium, soymilk, general–purpose medium plus, lactic medium, LAPTg broth, iso–sensitest media, etc. According to the American Society of Brewing Chemists [10], MRS is more preferable than Raka Ray, Barney–Miller Brewery, and Kirin–Ohkochi–Taguchi media since MRS gave higher colony counts of *Lactobacilli*. Vera *et al.* [11] carried out the quantification of lactobacilli type I sourdoughs in 11 types of media amongst which maltose MRS, MRS, SFM and Rogosa media presented 9.47, 9.43, 9.1 and 9.24  $\log_{10}$  cfu/g colony counts respectively; levels on the remaining MRS5, SDB, Vogel, FH, OH, LAMVAB, KCA media were 0.5  $\log_{10}$  cfu/g lower than maltose MRS and MRS media, which also appeared the most appropriate broth media for colony subculture. Saman *et al.* [12] reported that brown rice and rice bran are suitable substrates for the culture of the probiotic *L. plantarum*; they showed that all the media used in the study produced a similar growth of *Lactobacilli plantarum* ( $> 3 \log_{10}$  CFU/mL increment). Corcoran *et al.* [13] showed that probiotic LGG can use an exogenous oleic acid source to increase their acid survival, while the mechanism most likely involves the ability of increased membrane oleic acid to be reduced by  $H^+$  to stearic acid. In the present study, a modified media LGGM has been designed on the basis of the original formulation of MRS and by utilizing the characteristics specific to the LGG. In quantitative assays of the present study, LGGM showed a greater cfu than the MRS medium. The growth of pure LGG strain was significantly greater in LGGM, and the recovery rate of LGG released from fecal samples (after oral feeding) was significantly greater ( $P < 0.001$ ).

The composition of media influenced the antimicrobial activity of *Lactobacillus* strains. Raja *et al.* [14] noted antimicrobial activity of *Lactobacillus* against *E. coli*, *S. typhimurium*, *S. aureus* that were due to pH effect as a result of lactic acid production. On LGGM agar the strains of *E. coli*, *K. aerogenes*, *S. typhi*, *S. sonnei*, *P. vulgaris*, *P. aeruginosa*, *S. aureus*, *V. cholerae* failed to form colonies, but in MRS media at pH 6.5, LGG, *E. coli*, *P. vulgaris*, and *S. aureus* was found to grow; remaining test strains did not grow, thus, supporting the selectivity of the LGGM. Ogunbanwo *et al.* [15] showed that *L. brevis* strain inhibited *E. coli* and *Enterococcus faecalis* (*E. faecalis*), but did not inhibit *Candida albicans*, *Klebsiella* sp. The *L. rhamnosus* GP1 strain grown in MRS broth had the best inhibitory effect against wide spectrum of bacteria: *St. aureus*, *Ps. aeruginosa*, *E. coli*, *Bacillus subtilis*, *En. faecalis*, but it did not inhibit *Proteus vulgaris* and *Klebsiella pneumoniae* [16]. Teanpaisan *et al.* [17] reported that oral *Lactobacillus*



strains showed a strong inhibitory effect against *Streptococcus mutans* and *Streptococcus sobrinus*, as well as, gram-negative periodontal pathogens *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*; they showed that the inhibition was enhanced in acidic condition and 5% glucose. One dose ( $10^8$  cfu/mL infused into the vaginal tract) of the human *Lactobacilli fermentum* L23 strain colonized and persisted in the vaginal tract of the female BALB/c mice for 5 days, and the study indicated that the probiotic *Lactobacilli fermentum* L23 produced both preventative and curative effects on *E. coli* growth [18]. *Lactobacillus* GG exerts an antagonistic action against macrolide-resistant group A streptococci by inhibiting cell invasion [19].

The LGGM was prepared by adding the components of MRS media except the dextrose, by lowering the pH of the media, by adding antibiotics and a chromogenic indicator of sugar fermentation. Inclusion of yeast extract and Tween 80 (0.1%) in MRS medium increased the bacteriocin production of *L. rhamnosus* GP1 isolate [16]. Higher antimicrobial activity was shown by addition of yeast extracts (3.0%), NaCl (1.0%–2.0%), glucose (1.0 %) and Tween 80 (0.5%), while addition of tri-ammonium citrate, sodium acetate, magnesium sulphate, manganese sulphate and potassium phosphate had no effect on activity [15]. To improve the growth and efficiency of probiotic bacteria, selective sources of carbon and energy, in the form of prebiotic are introduced in the culture media. Saminathan *et al.* [20] showed that addition of prebiotics such as isomaltooligosaccharides in MRS broth supported good growth for 11 different types of *Lactobacillus* strains. Matijevic *et al.* [21] examined the influence of lactulose on the growth and activity of *Lactobacilli acidophilus* and *Bifidobacterium animalis* subsp. *lactis* and found that its addition in reconstituted sweet whey prolonged the time of fermentation in both bacteria species, but it did not influence the viable cells count at the end of fermentation. The Nx (40  $\mu$ g/mL) made LGGM inhibitory to most intestinal bacteria, including lactic acid bacteria that grow on MRS agar, but had no adverse effect on LGG recovery. Eventually if any intestinal lactic acid bacteria grew in LGGM, they formed colorless small colonies due to their inability to ferment sorbitol that were readily distinguished from those of LGG. The yellow color change produced by the LGG strain in the LGGM after 48 h of incubation was due to acid production from sorbitol in the presence of bromocresol purple [22]. A similar finding was previously reported for *L. plantarum* by Bujalance *et al.* [23].

The knowledge of antimicrobial susceptibility helps to explore the resistance pattern of probiotics as well as make use of combination of probiotics and antibiotics for the restoration of the normal intestinal flora [24]. Thus, antimicrobial susceptibility testing is necessary for probiotic culture and antimicrobial therapy [25]. Huys *et al.* [26] investigated the influence of MRS agar and iso-sensitest agar (ISA) on antibiotic susceptibility of *Lactobacillus* and observed that Am and tetracycline (Tc) produced larger zones; Gm, bacitracin and erythromycin (Er) produced smaller zones on MRS compared to ISA, while Tc formed

similar ZDI. LAPTg and MRS agars were used to study antimicrobial susceptibility in which all *Lactobacillus* strains grew at concentrations above 10  $\mu$ g/mL of chloramphenicol (Ch), aztreonam, Nf, Cp, ceftazidime, Cn, streptomycin (Sm) and kanamycin (Km), while 4 were sensitive to 1  $\mu$ g/mL vancomycin and all of them were resistant to 1 000  $\mu$ g/mL of metronidazole [24]. Upreti *et al.* [27] found resistance in *Lactobacilli* strains to the antibiotics Am, Cp, Gm, Nf, Ch, Er, Km, novobiocin, Sm, and teicoplanin. The LGG strain was interpreted as resistant to Nx, Pb, Co, Gm; intermediate resistance to Cm, Cn, Cp and sensitive to Lf, Am, Nf, based on the ZDIs, and MIC determination as has been represented in our study. The LGG strain exhibited a growth of 1.1  $\log_{10}$  cfu/mL to 7.89  $\log_{10}$  cfu/mL in presence of 0–10  $\mu$ g/mL Cm and Nf in MRS broth media, thus showing high sensitivity to Cm and Nf (Figure 3).

The growth of LGG on the MRS agar was noticed at pH 3 to 7, with 6.65 to 5.48  $\log_{10}$  cfu/mL respectively; maximum growth of 7.89  $\log_{10}$  cfu/mL was found at pH 4.5. The quantification of exogenous lactobacilli in faecal samples is frequently required for the evaluation of the intestinal colonization by probiotic bacteria. Some faecal bacteria that were able to grow on MRS plates at pH 6.5 were inhibited in LGGM by incorporation of low pH of 4.5 in the LGGM that helped in the better enumeration of LGG, a property characteristic of LGG. Aslim *et al.* [28] determined the effects of different culture conditions including pH on growth of *Lactobacilli delbrückii* subsp. *bulgaricus* and *S. thermophilus*. In a study by Sarika *et al.* [16] the bacterial growth was observed at pH 4, 5, 6, 7 and maximum growth was at pH 6; irrespective of the initial pH, the end-pH value of the culture broth decreased to 3.36. The bacteriocin production by *Lactobacillus* strain exhibited consistent stability over the wide range of pH (2.5 to 8.5) at room temperature with a reduction in activity at a high alkaline pH and highest activity in the pH range of 5.5 to 7.5. Maximal activity in culture medium was achieved at initial pH of 5.5, and incubation period of 48h at 30–37 °C [15]. The different strains of lactobacilli tolerated very low pH despite variation in their degree of viability; the growth of an *L. acidophilus* NCDC 291 strain showed decreased trend with decreasing pH in the range 3.5–4.5, with maximum cell count of 6.28  $\log$  cfu/mL at pH of 6.5 [29]. Verdenelli *et al.* [8] reported that *Lactobacillus rhamnosus* and *Lactobacillus paracasei* strains tolerated well low pH and bile acids, and could be recovered from stools of volunteers after the 3 months human feeding trial.

The current findings show that LGGM is suitable for detection of LGG in faecal samples after oral administration. This medium provides an excellent means to assess the intestinal colonization by LGG, whose probiotic potential has been well established particularly in reducing diarrheal disease [30, 31]. The present approach applied for making a selective and differential media specific for LGG culture could similarly be used in designing media specific for a particular *Lactobacillus* species having great probiotic potential.

## Conflict of interest statement

We declare that we have no conflict of interest.

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