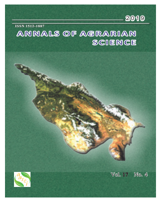
Annals of Agrarian Science

Journal homepage: http://journals.org.ge/index.php



First report on diversity of probiotics in Dambalkhacho, traditional fermented milk product of Georgia

T.Japaridze*, V.Baramidze , M.Vakhania , L. Kutateladze, E. Elizbarashvili, K. Didebulidze

Agricultural University of Georgia, Kakha Bendukidze University Campus, 240, David Aghmashenebeli Alley, Tbilisi, 0159, Georgia

Received: 22 November 2019; accepted: 12 February 2020

A B S T R A C T

Dambalkhacho is a traditional Georgian fermented milk product, protected under the patented brand name N 1585/071, manufactured from cottage cheese, with unique starter cultures, in the mountainous regions of Georgia. This study was undertaken in order to gain insight into the microbiota, in particular probiotic strains that take part in the manufacture and ripening stages of Dambalkhacho. Traditional cheeses are an important reservoir of microbial diversity that can have important biotechnological applications, starter cultures consisting of autochthonous bifidobacterial strains are of particular interest. To determine the strain identity and evaluate their probiotic potential, we made use of both culturing and the culture-independent methods of PCR. The main species isolated were *B. bifidum, B. longum and B. longum subsp. Infantis.* Culturing methods enabled the determination of a number of viable microorganisms from different microbial groups and their isolation for potential future applications in specific starter cultures. Strains were tested for tolerance to low pH and high bile concentrations, simulating the human gastrointestinal conditions. The probiotic strain B (KB 1.4); C (TB 2); D (TB 6.2.1); B (TL 8.2); D (TL 2.4.1) met the suggested initial count of 106 CFU/ml with brand C recording the highest at 9.19 ± 0.14 log CFU/ml. The higher bile concentrations resulted in lower growth in all the strains. After pH 3.0 treatment, B (KB 1.4); D (TB 6.2.1); D (TL 2.4.1) have also met the requirement of survival at 2 % bile concentration. Based on the overall technological aptitude of the tested strains B (KB 1.4); D (TB 6.2.1); D (TL 2.4.1) met the initial count requirement, and exhibited good acid and bile tolerance therefore being a potentially good source of probiotic additives.

Keywords: Probiotics, Bifidobacteria, Fermented milk, Dambalkhacho, Important reservoir, Traditional cheeses.

*Corresponding author: Tamar Japaridze: E-mail address: t.japaridze@agruni.edu.ge

Introduction

New forms and range of food products favoring nutritional and therapeutic aspects are being increasingly developed. There has been a phenomenal growth in consumption of foods containing functional ingredients, particularly in Western Europe, USA and Japan [1]. Bacteria belonging to the genus Lactobacillus are members of the lactic acid bacteria family (LAB), a broadly defined group characterized by the formation of lactic acid as the sole or main end product of carbohydrate metabolism. The administration of probiotic Lactobacilli stimulated indigenous Lactobacilli and the production of short-chain fatty acids. This alteration of the intestinal environment should contribute to maintain the host's health. They can be found mostly in fermented foods (yogurt, cheese, olives, pickles, salami, etc.), as well as in the oral cavities, gastrointestinal tracts (GIT) and reproductive tract of animals [2-4]. One of the first microorganisms colonizing infants gut is Bifidobacterium species, but the number and variety of Bifidobacterium species decreases with age, from childhood to old age. Bifidobacterium longum, B. breve, and B. bifidum are generally dominant in infants, whereas B. catenulatum, B. adolescentis and, as well as B. longum are more prevalent in adults. Moreover, bifidobacteria have been associated with the production of a number of potentially health promoting metabolites including short chain fatty acids, conjugated linoleic acid and bacteriocins [5]. The identification and enumeration of bifidobacteria has been reported by several research teams [6-9].

Dambalokhacho is a type of unique, traditional Georgian fermented milk products that is protected under the patented brand name N 1585/071. It has been produced for centuries in Georgia, predominantly in mountainous regions of the country. The production of the product is not industrialized thus, ancient fermentative microflora is maintained by isolated communities, and is harboring a wide pool for isolation of probiotic microorganisms. The search for new types of probiotics, from the microbiota of traditionally fermented foods especially exhibiting diverse composition of naturally occurring symbiosis of beneficial microorganisms, is the most promising pool providing novel strains [10] Increasingly, evidence is accumulating which shows beneficial effects of supplementation with bifidobacteria for the improvement of human health conditions ranging from protection against infection to different extra- and intra-intestinal positive effects [11-13]. Complex aproach is needed to identify probiotic properties of select strains. Several difierent methods have been reported but single system of tests has not been identified. Here we report the complex of experimental procedures, for identificar tin and evaluation of probiotic avtivity of bifidobacterial and lactobacillus strains isolated from spontaneously fermented, traditional milk products.

Recent studies have confirmed that bifidobacteria can protect from enteropathogenic infections through the production of acetate[14-15], which improves epithelial mediated intestinal defense, thus protecting the host against lethal infections. Furusawa et al. reported that a commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells, which have a critical role in suppression of inflammatory and allergic reactions [16]. These two studies are redefining the criteria for probiotic selection, providing us with a new means for evaluating the health beneficial effects of commensal bacteria.

Based on the above studies we intend to measure the acetate and butirate production of the selected strains to establish their probiotic activity. The acetate concentration will be evaluated by using EnzyChrom™ Acetate Assay Kit, which is designed for the quantitative determination of acetic acid or acetate and the evaluation of drug effects on acetate metabolism. Or with the same procedure as used for the butyrate, by gas chromatography, following the protocol developed and published by Richard E. Hiliman [17].

Materials and methods

Sample collection and bacterial strains

In total 30 samples were collected from specific ecological regions of Georgia, in small family farms or at local markets, from Pshavi, Ukana Pshavi Tianeti Pshavian villages. Samples were collected in sterile bags, labled, transported to the laboratory in cool boxes and stored at - 20 °C until further processing. All the samples were seeded onto specific selective media MRS, M17. BSM (sigma-aldrich), and reseeded up to 8 times, to obtain pure culture strains. 10 bifidobacterial strains were identified and selected for probiotic activity tests. The reference strains were purchased from DSMZ- Deutche Samml;ung von Mikrooganismen und Zellkulturen Gmbh. LactobacillusBulgaricus – ATCC 11842, Delbruck – ATCC 9649, Streptochochus tehrmophilus – ATCC 19258, Bifidobacterium Infantis– ATCC-15697, Bifodbacterium Longum-ATCC 15707, Bifidobacterium Bifidum- ATCC 2952. Gramm stained and visualized by light microscopy with Omax-wvr 1000 instrument [18].

DNA extraction and PCR

DNA was extracted using Milk DNA Extraction Kit (Norgen, Thorold, Ontario, Canada) bacterial DNA extraction kit, according to manufacturers instructions with slight modiffications as followed. Prior to DNA extraction 500 mg of dambalkhacho was homogenized in prelyzis buffer containing Tris 0.02 M, EDTA- 0.02 M. DNA quality and quantity was evaluated with nanodrop instrument ND-1000, NanoDrop Technologies, Inc.

Strain specific PCR was conducted for each pure strain using primers established by [19]. As well as designed by our team, using NCBI primer design toolConditions have been modified as follows; Initial heating for 2 min 95 °C, followed by 35 cycles consisting of denaturation 95 °C 15 s, annealing 57 °C 30 s, extension at 72 °C 40 s, and a final extension 72 °C 4 min. Amplicons were separated on 1 % agarose gel by electrophoresis and analyzed by ethidium bromide staining.

Probiotic activity tests

Strain survival in vitro conditions simulating the passage through the stomach and intestine was evaluated. All experiments have been carried out in triplicate.

Tolerance against Bile

MRS broth containing 0.3% and 2 % bile was inoculated with active overnight cultures. Culture with 0 % bile served as a control sample. The principle of assay was assessing the cell viability after exposure to bile salts (sigma) for 2, 4 and 6 h incubation with bile salts. Survival of strains were determined by pour plate counts of all the samples using 10-fold serial dilutions prepared in 0.1% peptone water. Viable cells were enumerated in 24 h anaerobic incubation and cell growth was monitored for growth every 3 h by measuring the absorbance at 620 nm [20].

Resistance to Low pH

Survival under low pH was evaluated based on methodology developed by Sahadeva and colleagues [21]. Active cultures were harvested by centrifugation and pelletswere washed once in phosphate-saline buffer (PBS, pH 7.2), re-suspended in PBS with pH adjusted to 1.5, 3.0 and 7.2 using 1M HCl (control with over 1.5 hour intervals) and incubated in MRS agar plates at 37˚C for 24 h.

Phosphoketolase assay

The ability of studied strains to transform fructose-6-phosphate into acethyl-phosphate and erythrose-phosphate by phosphoketolase, was studied according to the method developed by Zinedine et, all 2007.

Strains were grown in MRS broth for 24 h at 37°C, the culture was centrifuged and washed twice with a solution containing (PBS buffer 0.05% adjusted to pH 6.5 and added to 0.25% L-cysteine). Cells were then suspended in 2 ml of a lysozyme solution (10 mg/ml) and incubated a 37°C for 1.5 h followed by 1.5 h incubation at -20 °C. Afterwards 0.25 ml solutions containing 3 mg/ml of sodium fluoride and potassium iodoacetate 5 mg/ml was added, vortexed and incubated at 37°C for 30 min. Following incubation, 1.5 ml of a solution of hydroxylamine-HCl (13 g/100ml water, pH 6.5) was added and incubated at room temperature(RT) for 10 min. A reddish-violet color develops immediately with the addition of ferric chloride if the culture contains phosphoketolase activity. Accordingly, 3 ml of a solution of trichloroacetic acid (TCA at 15% w/v), 1.0 ml of 4N HCl and 1.0 ml of ferric chloride (FeCl, 6 H2O, 5% w/v in 0.1 N HCl) were added and incubated at RT for 10 min.

Acidification and Coagulation

Acidification and coagulation ability of LAB strains were assayed by inoculating 10% skim milk (RM1254, HiMedia, Mumbai, India) at 1% level and incubated at 37◦C for 28 h. Observation was made for commencement of clotting, followed by pH measurement [22-23].

Growth curve

The stock culture of each strain was separately prepared on MRS medium. Inoculated cultures were incubated at 37 ° C for 24 h and then stored in a refrigerator at 4 ° C. Batch submerged fermentation process was performed in separate flasks containing autoclaved milk as the substrate, incubations performed at 37 ° C. Cell number measured by specrophotometry at 620 nm wave length, measurements taken once a day for 3 days. Lactobacillus Bulgaricus – ATCC 11842 used as control.

Results and discussion

In total, 30 Dambalkhacho samples have been collected. Isolates have been characterized for cell morphology and Gram's character test. 18strains were selected for further experiments based on gram staining and microscopy. A number of bacterial isolates isolated from dambalkhacho in the present investigation were gram positive, branched and rod shaped, anaerobic, catalase negative, non-spore forming, which were identified as bifidobacteria (Fig. 1). Several different selective media for Bifidobacterial isolation and enumeration have been tested, the best results had been obtained with modified BSM media of Sigma (88517 Sigma-Aldrich BSM Agar). In the medium, we have increased the malt-dextrin concentration, which gives more carbon and energy for the selected strains.

After, initial tests the pure strains were subjected to further species identification procedures, by DNA extraction and 16s rDNA PCR analysis. In all the PCR amplification experiments the reference strains were used as positive controls. Identification of the isolated bifidobacterial strains to species was performed using PCR technique applying 16S rDNA-gene-targeted primers specific to *B. bifidum, B. longum* and *B. longum subsp. Infantis,* out of 18 strains with stringent molecular techniques only 5 were classified to *B. bifidum,* 4 *B. longum subsp. infantis* and 7 to *B. longum species* (Table 1).

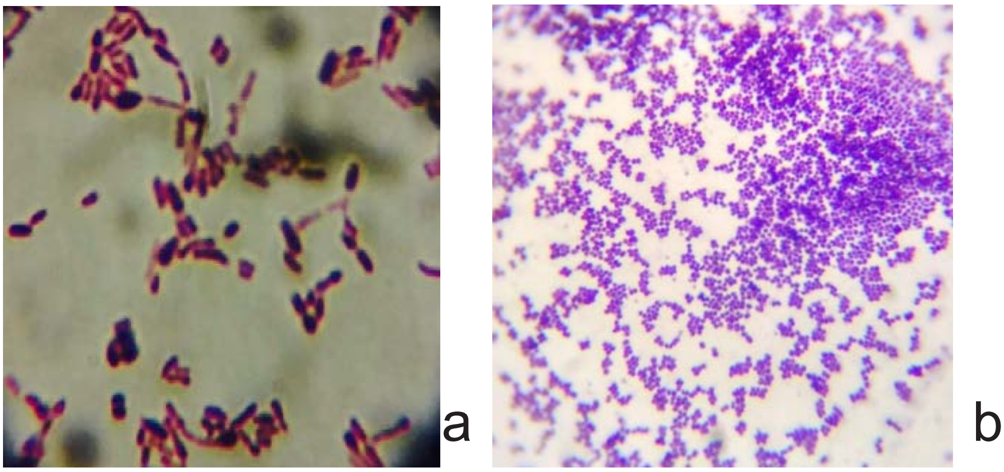


Fig. 1. *Representative pictures of select strains. Visualization by microscopy, x1000 magnification. Staining by Gramm stain. a) Sample seeded first time - mix culture; b) pure culture after 8 seedings*

all 3 species were isolated on MRS and BSM medium; In order to confirm the species specificity of isolated strains. Several PCR primer sets used (Table 2).

2 strains have not shown specificity of select primers and thus have not been assigned species. In this case, further studies are necessary. Strains belonging to

Table 1. *Names and the assigned species of the isolated strains*

Strains	Assigned species
A (KL10.2.1)	B. infantis
B (KL4.3.1)	B. infantis
C (TN5.7)	B. infantis
D (TN 5.3)	B. infantis
A (KB 12)	B.bifidum
B (KB 1.4)	B.bifidum
C (TB 2)	B.bifidum
D (TB 6.2.1)	B.bifidum
E (TUB 3.3.2.1)	B.bifidum
A (TL 1)	B. longum
B (TL 8.2)	B. longum
C (TL 5.3)	B. longum
D (TL 2.4.1)	B. longum
E (TL 3)	B. longum
F (6.5.5)	B. longum
G (2.1)	B. longum

Table 2. *Terget, Priner and Sequence*

Target	Primer	Sequence	(bp)
Bifidobacterium	g-Bifid-F	CTCCTGGAAACGGGTGG	549-563
	g-Bifid-R	GGTGTTCTTCCCGATATCTACA	
B. adolescentis	BiADOg-1a	CTCCAGTTGGATGCATGTC	279
	BiADOg-1b	TCCAGTTGACCGCATGGT	
B. bifidum	BiBIF-1	CCACATGATCGCATGTGATTG	278
	BiBIF-2	CCGAAGGCTTGCTCCCAAA	
B. breve	BiBRE-1	CCGGATGCTCCATCACAC	288
	BiBRE-2	ACAAAGTGCCTTGCTCCCT	
B. longum	BiLON-1	TTCCAGTTGATCGCATGGTC	831
	BiLON-2	GGGAAGCCGTATCTCTACGA	
B. infantis	BiINF-1	TTCCAGTTGATCGCATGGTC	828
	BiINF-2	GGAAACCCCATCTCTGGGAT	
B. lactis	Bflac2	GTGGAGACACGGTTTCCC	680
	Bflac5a	CACACCACACAATCCAATAC	

Some of the primers tested have shown crossreactivity to various other lactobacillus species (data not shown). Nonetheless, nonspecific PCR products were reduced, when annealing temperatures were increased to just below the maximum temperature at which no amplicons were produced. Primer sets selected during our investigations showed a considerable lack of specificity, even under our modified, more stringent PCR conditions. Experimental results of PCR are shown in Fig.2.

One of the most important criterion for probiotic selection is their tolerance to high acid levels, which is a determinant factor for the survival in the GI tract. The lowest pH in the GI tract according to Liong 2005 is 1.5 [21], [23], [28]. Whereas Leroy 2004, reports that pH 3.0 resistance is a threshold for best probiotic sources, and pH 7.2 is counted as control due to the constant probiotic counts [23]. Selected strains were incubated at different pH values of1.5, 3.0 and 7.2 (control) with over1.5 h intervals. Four strains, C (TB2); D (TB6.2.1); C(TL 5.3) and D(TL 2.4.1) have met the minimum initial count requirement asset by WHO/FAO2006.The acid tolerance for all isolates were the same where increasing level of acidity has negative impact on the viability of the probiotics (Fig. 3).

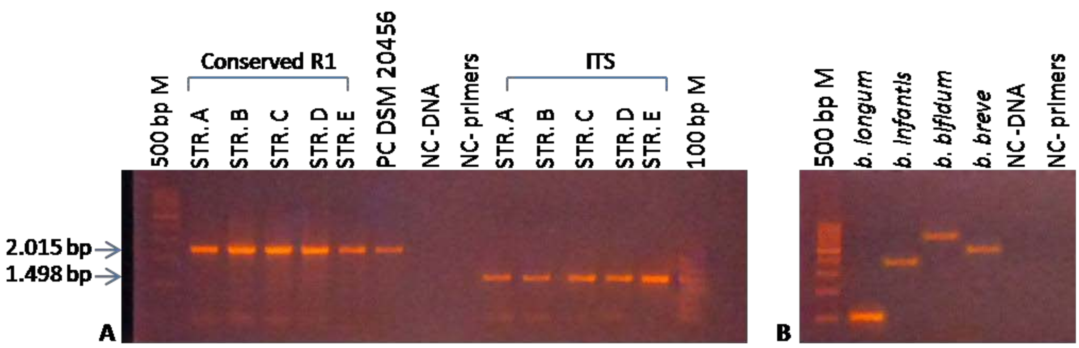


Fig. 2. *Representative agarose gel of amplification with PCR. A. Genus specific PCR Amplification using Bifidobacteria genus specific primers for ITS (g-Bifid-F; g-Bifid-R) and Conserver region 1 (LonU7; LonU8). Lane 1- molecular weight marker 500 bp. Lane 2-6 select strains, with the strain name indicated above the band. Positive control (PC+) reference strain. B. Multiplex PCR Lane 1-molecular weight marker 500 bp. Lane, 2 -5 control strains, with the strain species indicated above the band. Negative control (NC-) reaction without DNA or primers*

All strains have met the minimum requirement set by FAO/WHO (1 x 106 CFU/ml) except for strain E. The highest number of live probiotics was recorded by strain C with a count of 1.55 x 109 CFU/ mL and the lowest was recorded by strain E with a count of 2.40 x 105 CFU/ mL Judging by this initial count, strain A, B, C and D are considered good probiotic sources except for strain E (TUB 3.3.2.1). Amongst the reasons for the low count in strain E could be affected by the temperature during the fermentation process.. As a Brand E is strictly anaerobic, the oxygen that is dissolved in the product during manufacturing could stress the probiotics as too much oxygen will delay their growth [24]–[27]

Three selected strains showing the highest probiotic activity in previous tests have been subjected to cell viability and bile resistance tests for longer exposure times. Bile plays an important role in the physiology of intestinal bacteria. This is particularly important for probiotic bacteria, since their beneficial effects must be generated in their presence. It is known that the activities of intestinal bifidobacteria are deeply influenced by the presence of bile salts, and even some of them, such as cholesterol assimilation, have been directly correlated with bile salt metabolism in these bacteria.

To evaluate bile salt tolerance, viable cells were counted by measuring absorbance at 620 nm spectro-

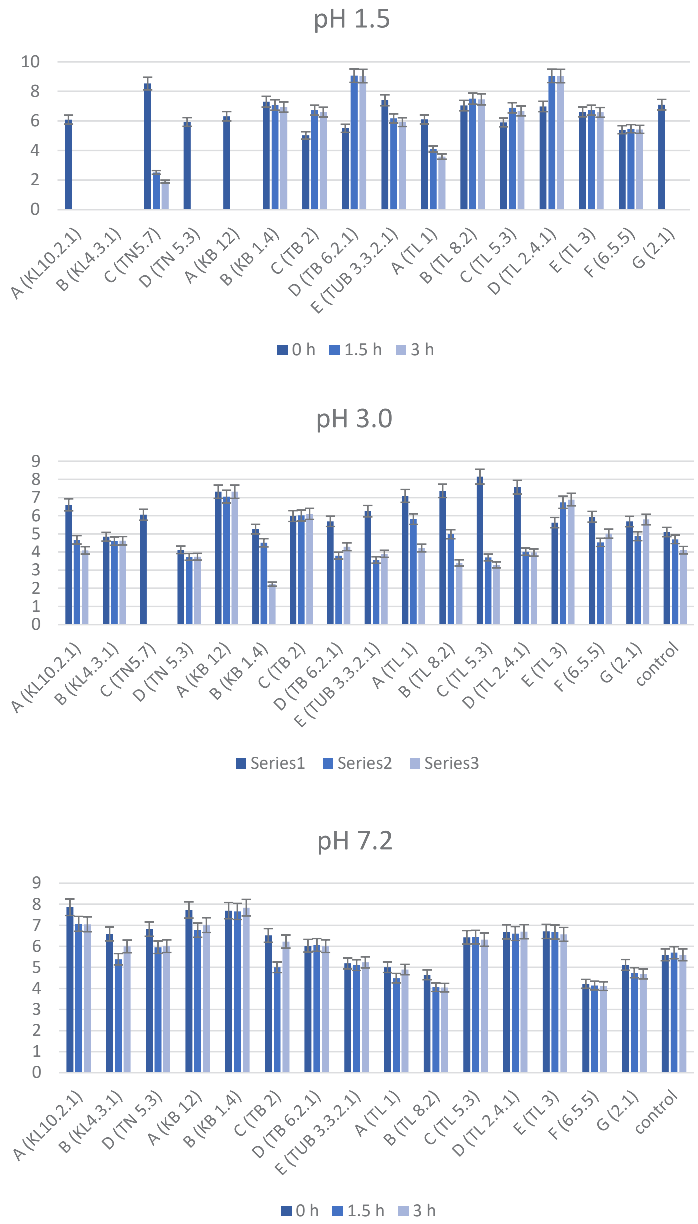


Fig. 3. *pH resistance of select strains. Total plate counts for five select strains on MRS agars at different pH values of 1.5, 3.0 and 7.2(control) over 1.5 hour interval*

photometrically using the method by [20]. The data shown in Fig 4 demonstrates, that all the select strains showed significantly higher resistance to bile salts compared to commercially available control strains.

increase in cell viability after 24 hours was 62% average among the select strains reaching its highest in strains A (KL10,2,1) whereas the viability increased by 91% after cultivating the cells for 72 hours, thus indicating the highest survival rate, *in vitro*, among the strains studied by the team. the mean values, derived from the experiment lead to the following observations:

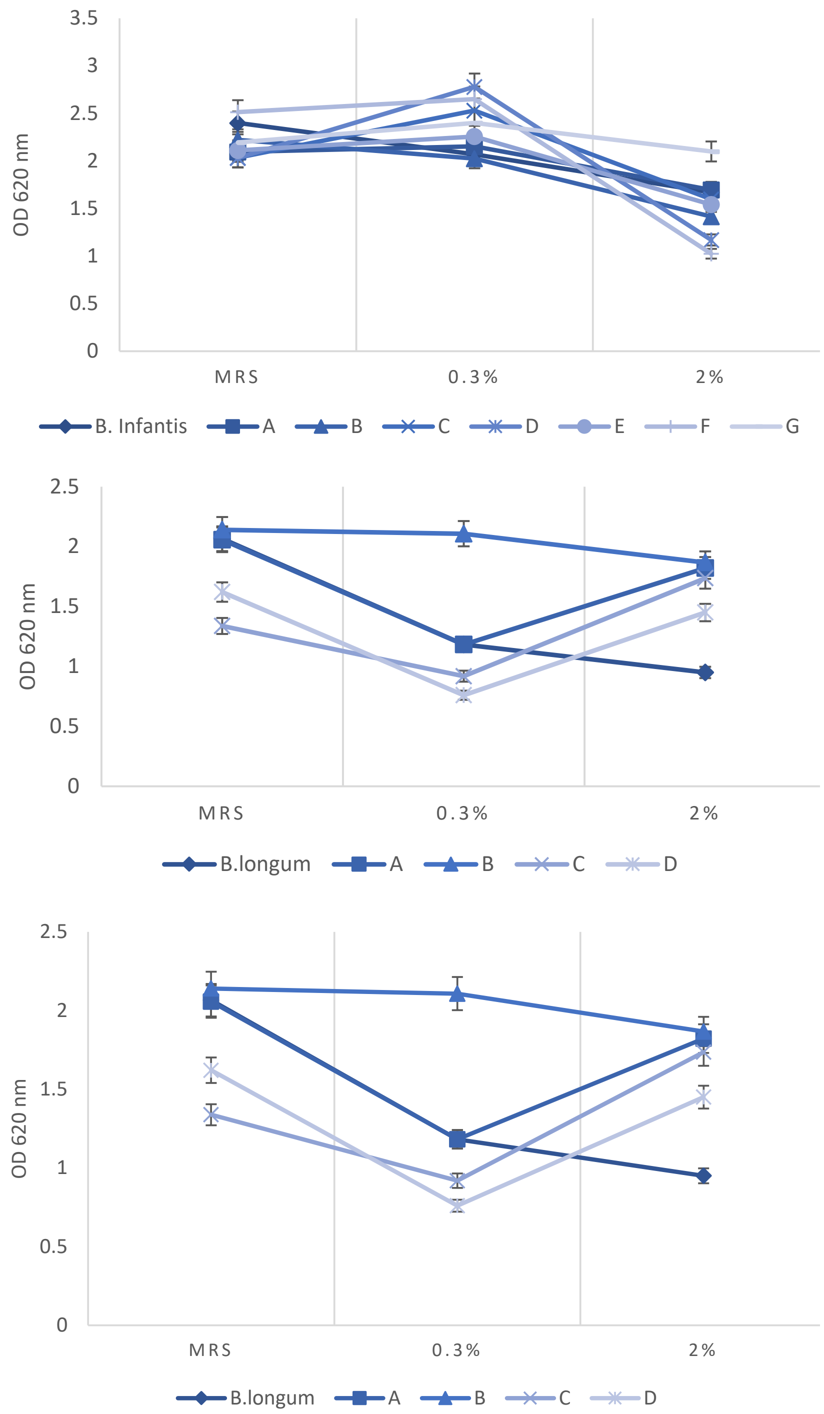


Fig. 4. *Bile salt resistance. Tolerance against bile salt (MRS, 0.15%, 0.3%) – OD620 nm values during 24 hours of incubation at 37°C. p < 0.05. results represent the mean, standard error pf three experiments (n¬=3)*

All bifidobacterial strains have shown a high acidification rate in the milk. During the fermentation we haven't seen the changes in shape and Gas production. The weakest congealing was detected in strains A (KB 12);D (TB 6.2.1)C (TL 5.3), results of growth and acidification Fig. 5. All other strains showed good structure formation and also very strong gas formation.

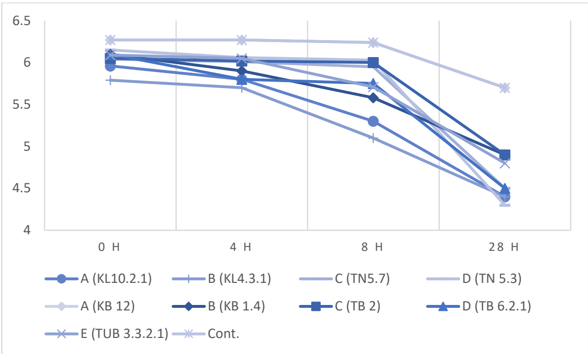


Fig. 5. *Milk acidification kinetics by select probiotic strains. All experiments were realised in the reconstituted milk at 37 °C. Commercial L. Bulgaricus – ATCC 11842 strain used as control.*

Tolerance to various pH and bile concentrations by simulating the human gastrointestinal pH and bile concentration (Fig. 4). The acid tolerance test was studied under pH 1.5 and 3.0 with 7.2 as control. The cell count for the acid tolerance test was obtained at an interval of 0, 1.5 and 3 hours respectively and was plated onto duplicate MRS agars to be incubated at 37C for 48 hours. All cells recovered after 3 hours of pH treatment were selected for bile tolerance test in MRS broth containing bile concentrations of 0% (control), 0.3% and 2.0% and cell counts were recorded after 24 hours of incubation. The probiotic strains in products A, B, C & D met the suggested initial count of 106 CFU/ml with brand C recording the highest at 9.19 ± 0.14 log CFU/ml. Strains in product A, B & C showed good tolerance to pH 3.0 and 7.2 recording a count of >106 CFU/ml after 3 hours with a range of 6.60 – 9.04 log CFU/ml. The higher bile concentrations resulted in lower growth of strains in all the brands. Upon pH 1.5 treatment, only brand C recorded growth in all bile concentrations. After pH 3.0 treatment, all brands except brand E met the requirement of survival at 0.3% bile concentration. Results showed probiotics in product A, B & C met the initial count requirement, and exhibited good acid and bile tolerance therefore being a potentially good source of probiotic.

Conclusion

Microflora of each Dambalkhacho, from difa ferent makers and different regions, varied considerably, based on preliminary exploration. Face tors such as milk, pH, and salt concentration may have contributed to the unique bacterial composition of each product, although no particular factor was determined to be responsible for differences in

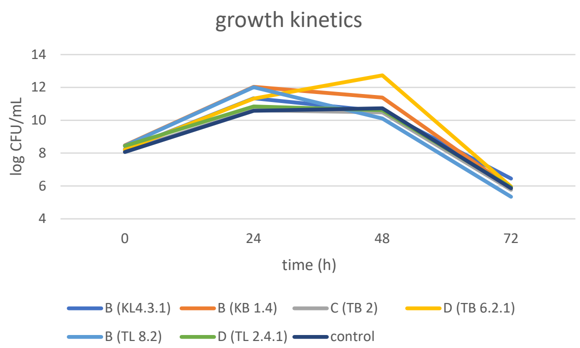


Fig. 6. *Growth kinetics of select probiotic strains during fermentation with six bifido strains. Each % culture by volume, with a final cell count of 8e9 log CFU/mL. Values are expressed as the mean AE standard batch was inoculated with 1deviation (n=3)*

abundance between the brands based on the limited available information. Pure strains of *B. bifidum, B. longum* and *B. longum subsp. Infantis* have been isolated from Dambalkhacho, collected from high mountainous regions of Georgia. All strains have been characterized microbiologically, and assigned species based on 16s variable region PCR aplification. Apart from 16 known strains the study resulted in 2 unassigned gramm positive, non-motile, catalase negative, rod shaped strains that require further investigation. Isolatedprobiotic strains in B (KB 1.4); C (TB 2); D (TB 6.2.1); B (TL 8.2); D (TL 2.4.1) met the suggested initial count of 106 CFU/ml with brand C recording the highest at 9.19 ± 0.14 log CFU/ml. The higher bile concentrations resulted in lower growth of strains in all the strains. After pH 3.0 treatment, B (KB 1.4); D (TB 6.2.1); D (TL 2.4.1) have also met the requirement of survival at 2 % bile concentration. Growth kinetic study also reviled, B (KB 1.4); D (TB 6.2.1) far exceeded control growth rates, of control strains. Based on the overall technological aptitude of the tested strains B (KB 1.4); D (TB 6.2.1); D (TL 2.4.1) met the initial count requirement, and exhibited good acid and bile tolerance therefore being a potentially good source of probiotic additive to the dambalkhacho.

Acknowledgements

The article is written on the results of the project implemented by financial support of the Shota Rustaveli National Scientific Foundation (Project 21 6924).

References

[1] S. Rezac, C. R. Kok, M. Heermann, and R. Hutkins, "Fermented foods as a dietary source of live organisms," Frontiers in Microbiology. 2018.

[2] M. De Angelis and M. Gobbetti, "Lactobacillus SPP.: General Characteristics," Ref. Modul. Food Sci., Jan. 2016.

[3] A. C. Ouwehand, S. Salminen, and E. Isolauri, "Probiotics: An overview of beneficial effects," Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol., 2002.

[4] K. J. Heller, "Probiotic bacteria in fermented foods : product characteristics and," vol. 73, no. 2 (2001) 374-379.

[5] N. B. Danneskiold-Samsøe et al., "Interplay between food and gut microbiota in health and disease," Food Res. Int., Jul. 2018.

[6] N. Klijn, A. H. Weerkamp, and W. M. D. E. Vos, "Identification of Mesophilic Lactic Acid Bacteria by Using Polymerase Chain Reaction-Amplified Variable Regions of 16S rRNA and Specific DNA Probes," vol. 57, no. 3390–3393, 1991, pp 11.

[7] J. Jany, G. Barbier, J. Jany, G. Barbier, and J. Jany, "Culture-independent methods for identifying microbial communities in cheese To cite this version : Culture-Independent Communities in Cheese Methods Identifying Microbial, 2011.

[8] H. Kwon, E. Yang, S. Yeon, B. Kang, and T. Kim, "Rapid identification of probiotic Lactobacillus species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA," vol. 239 (2004) 267–275.

[9] A. Galanis, Y. Kourkoutas, C. C. Tassou, and N. Chorianopoulos, "Detection and Identification of Probiotic Lactobacillus plantarum Strains by Multiplex PCR Using RAPD-Derived Primers," (2015) 25141–25153.

[10] M. Bright and S. Bulgheresi, "A complex journey: Transmission of microbial symbionts," Nature Reviews Microbiology. 2010.

[11] R. Tojo et al., "Intestinal microbiota in health and disease: Role of bifidobacteria in gut homeostasis," World Journal of Gastroenterology. 2014.

[12] M. Kleerebezem and E. E. Vaughan, "Probiotic and Gut Lactobacilli and Bifidobacteria: Molecular Approaches to Study Diversity and Activity," Annu. Rev. Microbiol., 2009.

[13] S. L. Long, C. G. M. Gahan, and S. A. Joyce, "Interactions between gut bacteria and bile in health and disease," Mol. Aspects Med., vol. 56 (2017) 54–65.

[14] S. Fukuda, H. Toh, T. D. Taylor, H. Ohno, and M. Hattori, "Acetate-producing bifidobacteria protect the host from enteropathogenic infection via carbohydrate transporters," Gut Microbes, 2012.

[15] S. Fukuda et al., "Bifidobacteria can protect from enteropathogenic infection through production of acetate," Nature, 2011.

[16] Y. Furusawa et al., "Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells," Nature, 2013.

[17] R. E. Hillman, "Simple, rapid method for determination of propionic acid and other short chain fatty acids in serum," Clin. Chem., 1978.

[18] T. J. Beveridge, "Use of the Gram Stain in Microbiology," Biotech. Histochem., 2001.

[19] M. G. Fortina, G. Ricci, A. Acquati, G. Zeppa, A. Gandini, and P. L. Manachini, "Genetic characterization of some lactic acid bacteria occurring in an artisanal protected denomination origin (PDO) Italian cheese , the Toma piemontese," vol. 20 (2003) 397–404.

[20] S. E. Gilliland and D. K. Walker, "Factors to consider when selecting a culture of Lactobacillus acidophilus as a dietary adjunct to produce a hypocholesterolemic effect in humans.," J. Dairy Sci., vol. 73, no. 4, (1990) 905–911.

[21] R. P. K. Sahadeva et al., "Survival of commercial probiotic strains to pH and bile," Int. Food Res. J., vol. 18, no. 4 (2011)1515–1522.

[22] C. Béal and G. Corrieu, "Viability and Acidification Activity of Pure and Mixed Starters of Streptococcus salivarius ssp. thermophilus 404 and Lactobacillus delbrueckii ssp. bulgaricus 398 at the Different Steps of Their Production," LWT - Food Sci. Technol., vol. 27, no. 1, (1994) 86–92.

[23] F. Leroy and L. De Vuyst, "Lactic acid bacteria as functional starter cultures for the food fermentation industry," Trends Food Sci. Technol., vol. 15, no. 2 (2004) 67–78.

[24] B. D. Towbin, Y. Korem, A. Bren, S. Doron, R. Sorek, and U. Alon, "Optimality and sub-optimality in a bacterial growth law," Nat. Commun. , vol. 8 (2017) 1–8.

[25] M. H. D. pd. Zwietering, I. Jongenburger, F. M. Rombouts, and K. van 't Riet, "Modeling of the bacterial growth curve.," Appl. Environ. Microbiol, 1990, vol. 56, no. 6 (1990) 1875–1881,

[26] T. A. Roberts and J. Baranyo, "A dynamic approach to predicting bacterial growth in food," Int. J. Food Microbiol., Vol.5, no 3 (1994) 125-142.

[27] G. N. Fatemeh Ardestani, F. Rezvani, "Evaluation of cell growth and substrate consumption kinetic of five different Lactobacilli in a submerged batch whey culture for lactic acid production," Int. J. Eng., 2016.

[28] M. T. Liong and N. P. Shah, "Acid and Bile Tolerance and Cholesterol Removal Ability of Lactobacilli Strains," J. Dairy Sci.,vol. 88, no. 1 (2005) 55–66.