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Lactic acid bacteria strains isolated from Kombucha with potential probiotic effect

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MATEI BOGDAN [1*], SALZAT JUSTINE[2*], DIGUȚĂ CAMELIA FILOFTEIA[1], CORNEA CĂLINA PETRUȚA[1], LUȚĂ GABRIELA[1], UTOIU ELENA ROXANA[1,3], MATEI FLORENTINA[1]
[1]*University of Agronomic Sciences and Veterinary Medicine Bucharest, Faculty of Biotechnologies, 59 Marasti Bld, Bucharest, district 1, Romania*
[2] *University Blaise-Pascale, Polytech Clermont Ferrand, France*
[3] *National Institute of Research and Development for Biological Sciences, 296 Splaiul Independenței, Bucharest, Romania*
[*] *equal contribution*
Corresponding author: *florentina.matei@biotehnologii.usamv.ro*

Abstract

Kombucha is an oriental traditional beverage made usually of sweetened tea fermented by a symbiotic consortium of bacteria and yeast embedded within a cellulose membrane. The beverage has been characterized for health benefits related mainly to the tea itself, but very few in relation to the microbial consortia. The objective of our work was to isolate lactic acid bacteria from a local kombucha source and characterize the strains for their probiotic potential. Five lactic acid bacteria have been isolated, characterized morphologically and by molecular tools; the results proved their belonging to the lactobacilli/lactococci group. Sequencing results lead to the conclusion that they all have 99% identity with strains of Pediococcus pentosaceus. The on-plate screening for bacteriocin production have been expressed on synthetic media only for three of the total five isolates. To complete the probiotic protential profiles of the isolates, they have been tested for their resistance to bile salts; only one isolate finally proved to have clear probiotic potential.

Key words: Kombucha, lactic acid bacteria, probiotic, bacteriocin, bile salts

1. Introduction

Kombucha (KBA) is an oriental traditional beverage made usually of sweetened green or black tea fermented by a symbiotic consortium of bacteria and yeast (SCOBY) embedded within a cellulose membrane. The fermentation process is static and the usual fermentation time is 7–21 days at room temperature (E. Loncar et al, 2006 [10]). Kombucha made of different tea sources has been studied for its health benefits related mainly to the tea itself. Such health benefits have been reported in relation to its anti-carcinogenic (R. Jayabalan et al., 2014 [7]) and anti-diabetic effects (A. Aloulou et al., 2012 [1]), as treatment for gastric ulcers (D. Banerjee et al., 2010 [2]) or high cholesterol (Z.W. Yang et al., 2009 [20]). Regarding the correlation between KBA microbial diversity and its health benefits very few reports have been issued and refers to lactic acid bacteria as potential probiotics (N.O. Kozyrovska et al., 2012 [8]). Kombucha microbial consortium originated from different geographical regions of the world has been characterized based on both culture-dependent (D. Dutta and R. Gachhui, 2007 [4]; C.H. Liu et al.,1996 [9]; A.L. Teoh et al., 2004 [17]) or culture-independent by high-throughput tools (J.A.Marsh et al., 2014 [12]; S. Chakravorty et al., 2016 [3]). Generally, the consortium is dominated by acetic bacteria like *Komagataeibacter* sp. and *Gluconobacter* sp. and yeast

(J.Jarrell et al., 2000 [6]; R. Jayabalan et al., 2014 [7]; J.A. Marsh et al., 2014 [12]). Aside this two main microbial groups, lactic acid bacteria (LAB) have been reported in both kombucha layers, soup and pellicle, counting up to 30% (J.A. Marsh et al., 2014 [12]). Different LAB species have been identified in KBA, like *Lactobacillus* sp., *Lactococcus* sp. or *Lecunosctoc* sp. (J.A.Marsh et al., 2014 [12], S. Chakravorty et al., 2016 [3]). J.A.Marsh et al. (2014) [12] have reported that *Lactobacillus* and *Lactococcus* are predominant in kombucha pellicles and the most common corresponded to a community that were 99% identical to *Lactobacillus kefiranofaciens* subsp. *kefirgranum*. Still 34-35% of the total bacteria remains unclassified, as assumed by S. Chakravorty et al. (2016) [3].

LAB produce a wide range of antimicrobial metabolites which include organic acids, diacetyl, hydrogen peroxide, antibiotics and bacteriocins (K. Kanatany et al., 1995 [5], Macwana, S. J., & Muriana, P. M., 2014 [11], Mohankumar. A, Murugalatha. N, 2011 [13], A. Matei et C.P. Cornea, 2014 [21], D.S.E. atuira et M.E.Popa, 2015 [23]) as well as other mteabolites, like exopolysaccharides (S.S. Grosu Tudor and M. Zamfir, 2014 [22]. Bacteriocins are ribosomally synthesized, extracellularly released bioactive peptides which have a bactericidal or bacteriostatic effect on other (usually closely related) species (M. Zamfir et al., 2000 [19]).

According to the World Health Organization probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. LAB are already worldwide recognized as probiotics which have proven proprieties of improving nutrition, intestinal disorders, improving the immune system and optimizing gut ecology (Moroeanu V.I. et al., 2015 [14]). Probiotic LAB belong to the genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Lactococcus*, *Streptococcus, Pediococcus* and *Leuconostoc.* Within this group, *Lactobacillus* species are most commonly used group of microorganisms for their potential beneficiary properties as probiotics (R.K. Pundir et al., 2013 [15]).

According to N.O. Kozyrovska et al (2012) [8] Kombucha tea/mat may be a promising formulation of a probiotic/prebiotic for human involved in activities under extreme conditions (spatial or arctic expedition). The objective of our work was to isolate lactic acid bacteria from a kombucha consortium used industrially for the production of a commercial probiotic product based on pollen and characterize the isolates for their probiotic potential.

2. Material and methods

Kombucha preparation

The Kombucha SCOBY consortium has been procured from Medica Farmimpex S.R.L., Otopeni, Romania through the kindly help of Dr. Ionut Moraru. Kombucha tea suspension has been prepared starting from green tea leafs (*Camellia sinensis*). The sweetened tea (90 g sucrose/L and 6 g/L plant) has been fermented with Kombucha inoculum during 18 days at 28ºC.

Microorganisms

As controls in our studies have been used pure cultures of *Streptococcus thermophilus* ATCC 19258, *Lactobacillus acidophylus* ATCC 4356 and *Lactobacillus fermentum* ATCC 9338 provided by the microbial collection of the Faculty of Biology, Bucharest University.

Lactic acid bacteria isolation

Because in KBA consortium there are present yeast and other bacteria than LAB a specific method has been developed in our lab for their isolation. Sample of 5 days ferment KBA have been sterile filtered by a Millipore membrane of 0.45μm to separate yeast from bacteria. Consequently, microaerobic conditions were applied in order to cultivate microaerophilic bacteria like LAB by a "sandwich" method developed in our laboratory. In

Petri dishes, 1mL of KBA filtrate was incorporated into the solid MRS (Man, Rogosa & Sharpe medium, Oxoid), then was added a thin layer of MRS agar 1% on it. Bacteria cultivation has been conducted at 37°C during 2-3 days. Specific isolated colonies have been transferred with the help of a sterile needle into liquid MRS and cultivated 24 hours at 37°C to obtain pure cultures.

Lactic acid bacteria identification

An initial morphological characterization has been applied to the bacterial isolates by Gram staining and optical microscopy visualization. Further, all the strains has been subject of molecular identification. DNA has been extracted by the use of Genomic DNA kit (Thermo Scientific) and its presence has been verified by electrophoresis with ethidium bromide. PCR amplification has been performed by the use of the primers LacF (5-AGCAGTAGGGAATCTTCCA-3) and LacR (5-ATTYCACCGCTACACATG-3). These primers are specific for the Lactobacillus group (L.E. Ritchie et al., 2010 [16]). PCR amplification was performed in a MultiGene thermocycler following the program: initial denaturation at 94ºC/2 minutes; 30 cycles of denaturation at 94ºC/15 seconds + annealing at 51ºC /15 seconds + extension at 72ºC / 30 seconds; final extension at 72ºC during 7 minutes. To separate and characterize PCR products, electrophoresis are run with 10μL of PCR sample spotting in 2% agarose gel containing ethidium bromide (14μg / μL). After a migration at 90 volts during 45 minutes, UV revelation was done. Meanwhile, the amplified fragments has been sequenced (Microsynth, Switzerland) and the obtained sequences have been analyzed by the use of EMBL database (www.srs.ebi.ac.uk). Also, theoretic digestion has been applied by the use of the Harry Mangalam's tacg 4.3 program (http://biotools.umassmed.edu/tacg4).

Screening for bacteriocin production

A qualitative screening method for bacteriocin production have been applied on the kombucha LAB isolates. As bacteriocin sensitive strain has been used *Streptococcus thermophilus* ATCC 19258. As positive control has been employed *Lactobacillus acidophilus* ATCC 4356 and as negative control *Lactobacillus fermentum* ATCC 9338. LAB isolates and controls have been cultivated in liquid MRS medium during 24 hours at 37ºC, followed by centrifugation (5000 rpm/10 minutes); the supernatant has been neutralized with 40% sodium hydroxide solution. In Petri dishes containing solid MRS 3μL of each supernatant has been added as spot followed by incubation at 30°C during 18 hours. The next day, 5mL 1% agar of MRS is mixed with 1mL of sensitive strain. This mixture is poured on the Petri dish with spots and incubated at 37°C during 24hours. If strains produce bacteriocin, an inhibition halo will appear around the spot (A. Mohankumar and N. Murugalatha, 2011 [13]).

Bile salt resistance

Kombucha LAB isolates have been tested for their resistance to different bile salts concentrations of 1.5% and 3 % recommended by other authors (Vamanu E. at al., 2012 [18] or higher, respectively 6%.. LAB isolates have been cultivated in MRS broth at 37ºC during 24 hours; after the cultivation have been centrifuged (5000 rpm/5 min), the pellet was resuspended in 200 μL of sterile water and was added in MRS broth supplemented with different concentrations of bile salts. The LAB growth have been estimated by measuring the turbidity/absorbance at 620 nm after 4 and 8 hours of static cultivation.

3. Results and Discussion

Generally, to obtain vinegar from kombucha, the fermentation should be longer than 14 days to let the acetic bacteria to develop sufficiently and to lead to the acetic acid accumulation. As reported by J.A.Marsh et al. (2014) [12] lactic acid bacteria are abundant in

the first days of the fermentation, this is why we have run the isolation activity in the first 5-7 days of the process. By the use of the improved "sandwich" method five punctiform white colonies, specific to LAB, have been isolated and transferred into pure cultures (S1, S2, S3, L3 and L5). All the cultures showed a positive reaction under Gram staining. Under optical microscopy different shapes have been noticed among the isolates, respectively S1 is a coccus, S2 and S3 are coco-bacilli, while L3 and L5 have a rod/bacillar shape.

After the PCR running with lactobacilli specific primers all the isolated strains has amplified same size fragment of 340bp (figure 1) which prove the belonging of all the isolates to the lactobacilli group. For the specie identification further sequencing has been proposed to be performed.

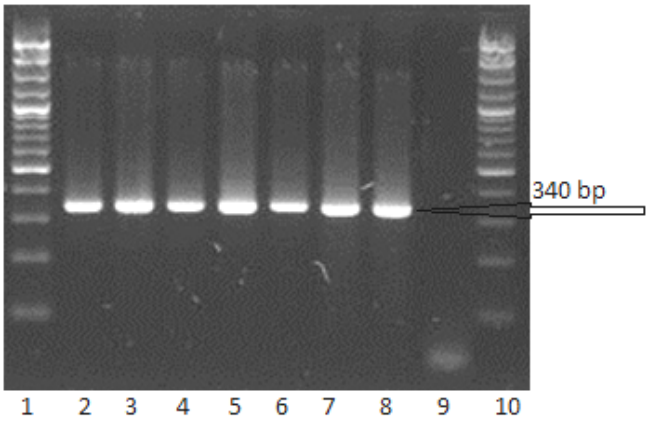

Figure 1. Amplified fragments with Lac F and Lac R primers (340 pb) specific to lactobacilli group (1&10-DNA 100 bp ladder; 2- S1; 3-S2; 4-S3; 5- L3; 6-L5; 7-*L.acidophilus;* 8- *L.fermentum*; 9- negative control)

The amplified fragments of 340 pb have been sequenced in both direction and the result have been analyzed by the use of Multiple Sequence Alignment, Clustal Omega tool, (http://www.ebi.ac.uk/Tools/msa/clustalo/). The analysis proved a 99% identify for all the isolates with strains belonging to *Pediococcus pentosaceus*; figure 2 presents the sequence in the case of L3 isolate. The presence of this specie in Kombucha has been reported already by Marsh et al. (2014).

AGCAGTAGGGAATCTTCCACAATGGaCGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCT
CTGTTGTTAAAGAAGAACGTGGGTAAGAGTAACTGTTtAcCCAGTGACGGTATTTAACCAGAAAGcCACGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGcGAGCGCAGGCGGTCTTTTAAGTCTA
ATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATTGGAAACTGGgAgACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGT
GTAgC**g**GGTGAAAT

Figure 2. Sequence of the amplified fragment of 340 pb with Lac F and Lac R primers for L3 lactic isolate

Several criteria are taken in account to call LAB as probiotic including activity antagonist against other microorganisms, resistance to acid, bile salt and phenol, adherence to gut epithelial tissue, cholesterol assimilation and lactose activity (R.K. Pundir et al, 2013 [15]). In our case we have tested the bacteriocin production by on-plate method. The results may be confirmed by the presence of functional genes involved in their production At this point, there are preliminary results (data not published) which lead to the conclusions that our isolates have amplified specific fragments for genes like *lclA* (lactococcin 972 encoding), *papA* (pediocin encoding), *pedA* (pediocin precursor), *NisZ* (nisine encoding) and *acdB* (acidocinB encoding). Data should be re-confirmed.

Bacteriocins produced by the probiotics inhibit the growth of sensitive bacteria. Contrary to antibiotics, bacteriocins are primary metabolites and they are produced with a maximum during the exponential phase or at the beginning of the stationary phase (M. Zamfir et al, 2000 [19]). This property is highly requested in food industries to avoid growth of pathogenic bacteria like Listeria monocytogenes.

Even if the presence of some bacteriocin functional genes can be proved by molecular screening, it is not sure if these genes are expressed, this is why an on-plate qualitative screening for bacteriocin production has been performed. Positive strains have developed an inhibition halo against the sensitive strain (*Streptococcus thermophilus* ATCC 19258) as described in material and method (figure 3). In the case of our strains three of the total of five LBA isolates have developed a halo similar in size with the positive strain (*Lactobacillus acidophilus* ATCC 4356), respectively S2, S3 and L5. Actually, there is a correlation with the amplified functional genes and the halo-formation as long as both S2 and S3 have amplified the encoding gene of acidocin B specific to *Lactobacillus acidophilus*.

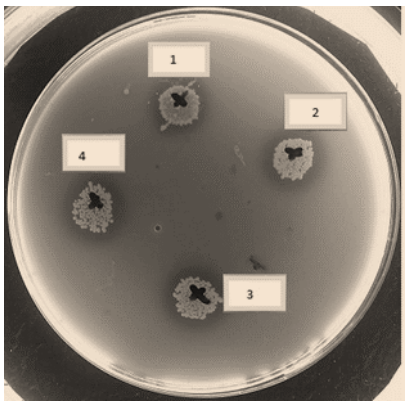

Figure 3. Aspects of bacteriocin screening on-plate. Positive strains are halo-forming. (1- S2; 2-*Lactobacillus acidophilus* - positive control; 3-S3; 4- L5)

Actually, LAB can be considered as probiotics only if they survive in human gut. This is why, testing the resistance to bile salts is one of the major test in the probiotic LAB screening (R.K. Pundir et al., 2013 [15]). Kombucha LAB isolates growth has been monitored at different bile salts concentrations in the first 8 hours of cultivation.

Tabel 2. Influence of different bile salt concentrations after 4 hours cultivation at 37ºC on the growth of lactic acid bacteria isolated from Kombucha microbial consortia

Bile salt conc. (%)	*Lactobacillus fermentum ATTC19258*	*Lactobacillus acidophyllus ATTC4356*	Isolate L3	Isolate L5	Isolate S1	Isolate S2	Isolate S3
1.5	1.95 ± 0.01	2.0 ± 0.01	1.95 ± 0.00	2 ± 0.02	2.06 ± 0.01	1.99 ± 0.01	1.0 ± 0.0
3	1.67 ± 0.05	1.8 ± 0.02	1.73 ± 0.01	1.65 ± 0.00	1.7 ± 0.05	1.06 ± 0.05	0.47 ± 0.01
6	1.34 ± 0.03	1.5 ± 0.05	1.6 ± 0.02	0.42 ± 0.05	1.61 ± 0.1	0.69 ± 0.01	0.19 ± 0.05

Note: Results are given in optical densitiy measured at 620 nm

In the first four cultivation hours it has been noticed that all the strains have similar growth and are resistant to a bile salts concentration of 1.5% (table 2). In the case of 3% (usual concentration in the humans) L3, L5 and S1 are highly tolerant (same as the controls), while S2 and S3 have been strongly inhibited (35%, respectively 60%). On 6% bile salts concentrations, after four hours, same S2 and S3 are strongly inhibited and L5 complete the series. It should be pointed out that during ingestion of probiotics, the most important hours are the six first. After eight cultivation hours all the strains have adapted to the medium and

showed the same growth levels. In terms of bile salts resistance, isolates S1 and L3 may be proposed as resistant potential probiotics.

4. Conclusions

In our effort to demonstrate the probiotic potential of the products based on fermentative process with kombucha consortium we have isolated five lactic acid bacteria (S1, S2, S3, L3 and L5). All these isolates are positive under Gram staining and have a cocco - bacillar shape. After amplifying their total DNA with lactobacilli specific primers, all the isolates have amplified a same size fragment of 340 bp, which prove their belonging to the lactobacilli group. The amplicons have been sequenced and in for all the isolates presents a 99% identity with strains belonging to *Pediococcus pentosaceus*.

The kombucha LAB isolates have been subject to different screening for their potential probiotic activity. The bacteriocin production has been positive by on - plate halo-formation for three of the five isolates (S2, S3 and L5). Furthermore, the isolates have been tested for their bile salts tolerance. Isolates L3, L5 and S1 proved to be high tolerant to 3% and 6% bile salts concentrations. In this context, has been proven that the kombucha as a whole has clear probiotic potential; taken into account all applied tests, isolate L5, which produce bacteriocin and is high tolerant to bile salt may be proposed as potential probiotic strains to be used for the production of different functional food or probiotic cosmetics.

Our findings are completing the picture of kombucha symbiotic consortium in the context of its future engineering, described by N.O. Kozyrovska et al. (2012) [8] to interact directly with the human host in new ways (catabolism of cholesterol, production of valuable biologicals in gut, etc.) on the basement of deciphered individual gut microbiome.

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