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Mutagenesis and Protoplast Fusion for Enhanced Bacteriocins Production

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Abstract

Background and Objective: Induced mutagenesis is widely used to produce novel mutants with improved productivities. Ethyl methane-sulfonate-induced mutagenesis followed by intergeneric protoplast fusion were used to develop lactic acid bacterial strains with high antimicrobial activities.

Material and Methods: The antagonistic activities of seven LAB strains were assessed against seven indicator microorganisms using well diffusion assay. The highest two producers were subjected to ethyl methane-sulfonate mutagenesis followed by intergeneric protoplast fusion. Selection of the mutants and the fusants from the suggested fusion cross was based on the responses to different antibiotics.

Results and Conclusion: *Lactococcus lactis* subsp. *lactis* and *Bifidobacterium longum* showed the highest antimicrobial activities against most of the indicator microorganisms. Such activities were achieved at pH 2.0 and dramatically decreased by increasing the pH level. Ethyl methane-sulfonate-induced mutagenesis resulted in thirty mutants, four of which exhibited higher activities than their wild type parental strains (two for each parent). In an attempt to increase such activity, intergeneric protoplast fusions between LM 13 (resulting from *Lactococcus lactis* subsp. *lactis)* and BM 4 (resulting from *Bifidobacterium longum*) mutants were carried out. Twelve fusants were obtained. Interestingly, one fusant (F1) showed an increase in antimicrobial activity, compared to its parental strains. An increased range of 58.1 to 345.7% compared to the parental strain *Lactococcus lactis* subsp. *lactis* and a range of 51.5 to 168.5% for the second parental strain were noticed. The LM 13, LM 6, BM 4 and BM 12 mutants and the F1 fusant can be used in the preservation of food products.

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1. Introduction

Lactic acid bacteria (LAB) play important roles in various fermented foods, including dairy, meat and vegetable products. LAB strains are able to secrete many inhibitory compounds such as organic acids (lactic and acetic), ethanol, diacetyl, H_2O_2 [1] and bacteriocins [2]. Bacteriocins are small peptides that are stable in a wide range of pH and temperatures. They are common examples of natural antimicrobial agents that can be used in food systems to extend product microbial - shelf life [3], and are generally considered as safe (GRAS) by the United States Food and Drug Administration (US FDA) [4]. Bacteriocins are generally divided into Class I (post-translationally modified peptides, lantibiotics with less than 28 amino acids small membrane-active peptides (<5 kDa), linear or globular peptides), Class II (unmodified peptides, non-lanthionine, 30-60 amino acids (<10 kDa), always exhibit unique properties of heat tolerance, and positive charges), and Class III (large proteins (>30 kDa), heat unstable). For example, Class I bacteriocins operon, like nisin, includes a structural gene, genes that give accessory proteins responsible for cell modifications (e.g. proteolysis, command peptide processing, dehydration, cyclization), transport genes (that encode the ABC superfamily transport proteins for transmission peptides), regulatory genes, and immunity genes that confer resistance to the producer strains [5]. Bacteriocins can kill or inhibit many food-borne path-

ogenic and spoilage bacteria such as: *Salmonella typhimurium*, *Listeria monocytogenes*, *Staphylococcus* spp., *Escherichia coli*, *Yersinia* spp. [6-8] as well as spore-forming bacteria of Bacillus and Clostridium genera [9]. Bacteriocins-producing LAB strains have been used as probiotics and bio-preservatives in food stuffs such as meat products [10,11], dairy products [12,13], fresh fish, sea-foods [14,15], salads and vegetables [6,16] and as natural preservatives in food packaging [17,18].

In nature, mutations occur randomly at extremely low rates (one mutant cell per 10^6 to 10^7 cells per generation). Mutations can result in changes in the nucleotide sequences of DNA (e.g. point mutation, double mutation, deletion and insertion) due to the expose to different mutagenic agents. Induced mutagenesis is widely used for producing novel mutant strains with improved phenotypes. The most common used mutagenic chemicals include: ethyl methane-sulfonate (EMS), methyl methane-sulfonate, nitrous acid and nitrosoguanidine (NTG) [19]. The most mainstream mutagen utilized to improve bacterial strains is EMS, which ordinarily produces assortments of point changes in the bacterial DNA. Via these point changes, extraordinary mutants are created at increased levels in correlation with the regular changes to upgrade the bacteriocin production [20,21]. Furthermore, ultraviolet (UV) and gamma irradiation are used as physical mutagenic agents [22]. They have already been used to improve the technological and physical characteristics of LAB. To increase lactic acid (LA) production, various mutagenic chemicals have been used such as: nitrous acid for *Lactobacillus delbrueckii* [23] and EMS for *L. pentosus* [24]. However, UV irradiation has increased the LA production and antimicrobial activity of *L. fermentum* against *Escherichia coli* [25]. Moreover, heavy ion irradiation was successfully used to increase LA production of *L. thermophilus* [26], while gamma irradiation was utilized by Abdallah et al. [27] to increase the antimicrobial activity of *L. acidophilus* and *Streptococcus thermophilus* against the antibiotic resistant *Salmonella* sp.

An important target of biotechnological studies includes the engineering of improved microbial cells for the synthesis of industrially valuable metabolites [28]. To achieve this target, different methods such as protoplast fusion and genetic engineering are usually employed. Protoplast fusion is an easy and cost effective technique that allows the exchange of entire genomes, through genetic recombination process between related or unrelated genera of microorganisms [19]. Protoplasts can be isolated from bacterial cells by digesting the cell walls with lysozyme in the presence of osmotic stablizers. The fusion of protoplasts occurs by chemical phosgens such as polyethylene glycol (PEG). This technique has great potentials in improving industrial strains through the transfer of genes between the parental strains in a random and non-specialized way, reversing the transfer by genetic engineering. The protoplast fusion technique can be utilized by developing a robust plan to isolate and develop fusant strains with specific and important productive characteristics [20]. It has been used to increase LA production of *L. rhamnosus* by 3.1 [29] and 1.8-folds [30] and *L. fermentum* strains by 4-folds [31] using intraspecific fusion. The intergeneric fusion between *L. delbruckii* and *Acetobacter pasteurianus* increased the yield of LA production by 5-folds [32]. Antifungal activity of *L. plantarum* has been improved by 3 rounds of protoplast fusion with *L. helveticus* [33]. The antifungal activities of the resulting fusants (F3C2 and F3A3) reached 1.92 and 2.00-fold, respectively, compared to parental strains. Intergeneric protoplast fusion has rarely been used to improve production of bacteriocins by LAB. So, this study was carried out to investigate antimicrobial activities of various LAB and improve these activities using chemical mutagenesis with EMS followed by protoplast fusion.

2. Materials and Methods

2.1. Materials

MRS broth, Brain Heart Infusion (BHI) broth, Plate Count Agar (PCA), different antibiotic disks and anaerobic jars were purchased from Oxoid (UK). Skim milk (SM) was obtained from the Dairy Science Department, Faculty of Agriculture, Cairo University, Giza, Egypt. Egg white lysozyme, mutanolysin enzymes, PEG 6000, EMS and 2[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) were purchased from Sigma- Aldrich (St. Louis, MO, USA).

2.2. Methods

2.2.1. Cultivation of lactic acid bacteria

The LAB strains (Table 1) were separately cultured in MRS broth for 48 h at 37 ºC in anaerobic conditions and stored at 4 ºC until use. Strains were monthly recovered from the refrigerated stock for 3 consecutive days at 37 ºC in bottles containing 100 ml of sterile SM (121ºC, 15 min). Indicator bacteria (Table 2) were separately cultivated in BHI broth at 37 ºC for 24 h and recovered monthly.

2.2.2. Antimicrobial activity assay

The antimicrobial activity assessment was carried out using well diffusion assay as described by Bazaraa et al. [15]. Briefly, 0.1 ml of each indicator microorganism (24 h old) containing 1.0×10^7 CFU ml^{-1} was spread plated over the surface of PCA medium and three wells (5 mm in diameter) were made using the top of sterile Pasteur pipette. Wells were filled with 50 µl of each two-days cultivated LAB strain in SM. Plates were incubated at 37 ºC for 48 h and the inhibition zones around the wells were measured in mm.

Table 1. The lactic acid bacteria and their sources

Strain	Code	Source
Lactococcus lactis subsp.*lactis*	1821	NRRL, Northern Regional Research Laboratories, Peoria, IL, USA
Lactobacillus rhamnosus	1937	
Lactobacillus casei	441	
Pediococcus acidilactici	14858	
Bifidobacterium bifidum	41410	
Bifidobacterium longum	15708	ATCC, American Type Culture Collection, Rockville, Maryland, USA
Streptococcus thermophilus	-	STS, Chr. Hansen, S lab, Denmark

Table 2. The indicator microorganisms and their sources

Strain	Code	Source
Bacillus cereus	33018	ATCC, American Type Culture Collection, Rockville, Maryland, USA
Escherichia coli	35218	
Salmonella typhimurium	14028	
Staphylococcus aureus	25923	
Listeria monocytogenes	7644	
Bacillus subtilis	765	NRRL, Northern Regional Research Laboratories, Peoria, IL, USA
Klebsiella pneumoniae	49	FS, Food Science Department, Faculty of Agriculture, Cairo University, Giza, Egypt.

2.2.3. Effect of pH on the antimicrobial activity

Active 24 h old LAB strains were separately grown in SM for 48 h at 37 ºC. The pH of each culture was then adjusted to different pH levels (2.0, 4.0 and 6.0) using either 1 N HCL or NaOH and the antimicrobial activity was assessed as previously described.

2.2.4. Ethyl methane-sulfonate mutagenesis

Five ml of 24 h old LAB strains separately cultivated in MRS were centrifuged (5.600 x g, 4 ºC, 3 min). Cells were resuspended in 5 ml of sodium phosphate buffer (pH 7.0, 50 mM) and then EMS was added to the suspension to achieve a final concentration of 200 mM. Tubes were agitated (100 rpm) at 30 ºC for 20, 40 and 60 min. Then, a 500 µl of sodium thiosulphate (0.4 M) was added to tubes to neutralize the EMS. Cells were harvested by centrifugation at (5,600 x g, 10 min) at 4 ºC and washed twice with the same buffer. The cell pellets were resuspended in phosphate buffer (pH 7.0, 50 mM), and appropriate dilutions (10^{-1} to 10^{-3}) were plated on the surface of MRS plates. Then, various antibiotic disks were placed on the surface of these plates and incubated at 37 ºC for three days [20,21].

2.2.5. Selection of mutants

Initial antibiotic sensitivity tests [34] were carried out on wild type strains using discs containing the following antibiotics with specific concentrations (µg/disc): amoxicillin, 25; chloramphenicol, 10; colistin, 25; doxycycline, 30; gentamycin, 10; polymyxin B, 300; rifampicin, 5; streptomycin, 10; sulphamethoxazole, 500 and tobramycin, 10. The most effective antibiotic disc was used for the selection of resistant mutants. Antibiotic-resistant mutants were collected from the nearest area around the discs (inhibition zones) and transferred to MRS plates for further studies.

2.2.6. Protoplast fusion

2.2.6.1. Protoplast formation buffer

The protoplast formation buffer (PB) [35] contained: $MgCl_2$, 1 mM; raffinose, 300 mM; gelatin, 0.5% and HEPES (pH 7.0, 20 mM).

2.2.6.2. Lactic acid bacteria carrying medium

The Lactic acid bacteria carrying medium (LCM) described by Efthymiou and Hansen [36] was utilized and contained (g l^{-1}): glucose, 20; trypticase peptone, 10; yeast extract, 5; tryptose, 3; K_2HPO_4, 3; KH_2PO_4, 3; tri-ammonium citrate, 2; sodium acetate, 1; Tween-80, 1 and l-cysteine-HCL H_2O, 0.2. The pH was adjusted to 6.8. For each liter of this medium, 5 ml of salt solution contains: (g/100 ml): $MgSO_4.7H_2O$, 11.50; $FeSO_4.7H_2O$, 1.68 and $MnSO_4$. $2H_2O$, 2.40 were added.

2.2.6.3. Protoplast regeneration medium

The protoplast regeneration medium **(**PRM) was similar to LCM without the addition of Tween 80, and supplemented with the following ingredients**:** 1% glucose; 0.5% bovine serum albumin (BSA) (2.5%, w v^{-1}); 2.5% gelatin; 2.5% $MgCl_2$; 25 mM $CaCl_2$; 300 mM raffinose and 2% agar [35]. The PRM was prepared as follow: raffinose and gelatin were dissolved in a portion of LCM (without Tween-80) with constant heating (50 °C in water bath) and stirring. Then, 2% agar was added to this solution and sterilized at 121 ºC for 15 min. Glucose, BSA, and salts were dissolved in the rest of LCM (without Tween-80), sterilized by filtration (0.45 µ Millipore filter) and directly added to the medium before pouring at 50 ºC.

2.2.6.4. Selection of the proposed cross

To perform protoplast fusion, antibiotic sensitivity test [34] was first carried out on parental strains, as negative controls, using different discs with specific antibiotic concentrations (µg/disc): ampicillin, 10; chloramphenicol, 30; erythromycin, 15; kanamycin, 30; lincomycin, 2; rifampicin, 30; streptomycin, 10 and tobramycin, 30. Based on the results, the adequate protoplast crossing was selected.

2.2.6.5. Protoplast fusion technique

Protoplast fusion was carried out according to Chassy [37]. Ten ml of MRS broth were inoculated with each parental strain and incubated overnight at 37 ºC. Then, 1.5 ml of the culture were sub-cultured into 50 ml of LCM medium containing: 0.1% glucose; 1% glycine and 20 mM threonine, and grown for 4 h at 37 ºC. Cells were then harvested, washed with PB and resuspended in 2 ml of the same buffer containing 400,000 U of lysozyme and 50 U of mutanolysin. Cells were incubated at 37 ºC with gentle agitation (100 rpm). Protoplast formation was microscopically verified using phase-contrast microscope. Cell wall removal and protoplast formation were observed within 1 h of incubation. Protoplasts were harvested by centrifugation at 4,000 x g for 10 min at 4 ºC and resuspended in PB. Two protoplast suspensions from different parental strains were mixed at 1:1 ratio, and then harvested by centrifugation at 4,000 x g for 10 min at 4 ºC. Supernatants were discarded and pellets were suspended in 1 ml of PB containing 100 µl of 40% PEG 6000 for 6 min. Suspension was centrifuged and pellet was resuspended in 3 ml of PB. Then, 5 ml of soft PRM (0.4% agar) medium at 45 ºC was mixed with 0.5 ml of protoplast suspension, plated immediately onto the surface of solid PRM agar plates and incubated at 37 ºC. After transferring the selected antibiotic discs, plates were incubated at 37 ºC for three days. Resistant fusants were collected, sub-cultured, tested for antimicrobial activity and stored at 4 ºC.

2.2.7. Statistical analysis

Statistical comparisons were carried out using one way Analysis of Variance utilizing the Costate software [38] and expressed as mean±standard deviation. All experiments were performed in triplicate.

3. Results and Discussion

3.1. Antimicrobial activity of lactic acid bacteria

The antagonistic activities of the tested LAB strains cultured in SM (initial pH of 2.0) for 48 h against selected indicator microorganisms are shown in Table 3 as diameter (mm) of clear zones. Results indicated that all used indicator microorganisms were variously inhibited by the bacteriocins produced by all the tested LAB. Data (Table 3) revealed that the highest antimicrobial activities belonged to bacteriocins from *Bifidobacterium* (*B.*) *longum* and *L. lactis* subsp. *lactis*, where the inhibition zones for each were the highest in 71.4% of the indicator microorganisms.

Table 3. Antagonistic activities of lactic acid bacteria

LAB*	Indicator microorganisms						
	Staphylococcus aureus	*Salmonella typhimurium*	*Bacillus cereus*	*Bacillus subtilis*	*Escherichia coli*	*Klebsiella pneumonia*	*Listeria monocytogenes*
	Inhibition zone (mm) ± SD						
Bifidobacterium longum	**10.3^{a}_{a}± 0.00	8.3^{a}_{b} ± 0.00	8.3^{a}_{b} ± 0.00	7.6^{a}_{c}± 0.00	7.3^{b}_{d} ± 0.00	5.4^{a}_{f} ± 0.00	6.6^{d}_{e}± 0.00
Streptococcus thermophilus	9.6^{b}_{b}± 0.41	6.6^{b}_{d} ± 0.02	6.6^{b}_{d} ± 0.06	7.6^{a}_{c}± 0.01	11.3^{a}_{a}± 0.03	5.6^{a}_{e} ± 0.00	7.6^{c}_{c} ± 0.01
Lactococcus Lactis	5.5^{c}_{d}± 0.00	8.6^{a}_{b} ± 0.00	7.8^{a}_{c} ± 0.00	3.5^{b}_{e}± 0.00	11.0^{a}_{a}± 0.00	5.3^{a}_{d}± 0.14	10.5^{a}_{a} ± 0.00
Lactobacillus casei	4.3^{d}_{b} ± 0.01	5.2^{c}_{a} ± 0.02	3.3^{e}_{c} ± 0.03	3.1^{cd}_{c}±0.00	4.5^{e}_{b} ± 0.05	4.5^{b}_{b} ± 0.03	5.2^{e}_{a}± 0.04
Pediococcus acidilactici	3.6^{e}_{d} ± 0.02	5.0^{c}_{b}± 0.02	5.2^{c}_{b}± 0.01	3.2^{c}_{e} ± 0.01	6.6^{c}_{a}± 0.03	3.5^{c}_{d}± 0.03	4.2^{f}_{c}± 0.01
Lactobacillus rhamnosus	3.5^{e}_{d} ± 0.00	5.3^{c}_{b}± 0.00	4.0^{d}_{c} ± 0.01	3.3^{c}_{d} ± 0.01	4.9^{de}_{b}±0.00	4.6^{b}_{bc}± 0.01	9.1^{b}_{a}± 0.19
Bifidobacterium bifidum	3.4^{e}_{c} ± 0.01	4.0^{d}_{b} ± 0.03	3.4^{e}_{c}± 0.01	3.0^{d}_{c} ± 0.00	5.4^{d}_{a} ± 0.01	4.3^{b}_{b}± 0.00	5.4^{e}_{a}± 0.03

*LAB strains were allowed to grow at 37 ºC for 48 h in SM. The pH of the culture was then adjusted to 2.0.
**Means followed by different superscripts (within columns) and different subscripts (within rows) are significantly different (p<0.05).

In antagonistic activity, *Streptococcus thermophilus* followed *B. longum* and *L. lactis* subsp. *lactis*, where it showed the highest activity against 42.9% of the indicator microorganisms. On the other hand, *B. bifidum* was the least in its antimicrobial activities. *Staphylococcus aureus* was most sensitive (10.3 mm) against bacteriocins from *B. longum* while *Salmonella typhimurium* and *Bacillus cereus* were most sensitive to bacteriocins of *B. longum* and *L. lactis* subsp. *lactis* with no significant differences (p<0.05) between them. Bacteriocins of *B. longum* and *Streptococcus thermophilus* equally (p<0.05) inhibited (7.6 mm each) *Bacillus subtilis*. Similarly, *Streptococcus thermophilus* and *L. lactis* subsp. *lactis* showed the same effect (11.3 and 11.0 mm) with no significant differences on *E. coli*. Bacteriocins of *B. longum*, *Streptococcus thermophilus* and *L. lactis* subsp. *lactis* equally inhibited (5.4, 5.6 and 5.3 mm, respectively) *Klebsiella pneumonia*. Finally, *Listeria monocytogenes* was mostly inhibited by bacteriocins of *L. lactis* subsp. *lactis*. Bazaraa et al. [15] reported similar results where *L. lactis* subsp. *lactis* was the most effective tested LAB in inhibiting selected food pathogenic and spoilage microorganisms. Antimicrobial activities obtained from LAB were seen against Gram-positive and Gram-negative bacteria (Table 3). Similar results were reported by Nomoto [39]. However, it is well known that nisin is generally active against Gram-positive bacteria while it is inactive against Gram-negative bacteria. This is linked to the resistance of the outer membranes of the Gram-negative bacteria which prevents this bacteriocin from entering the cells [40]. Such obtained activities against the Gram-negative indicator bacteria could be due to the synthesis of organic acids, diacetyl, hydrogen peroxide and possibly other bacteriocins [41]. Since *B. longum* and *L. lactis* subsp. *lactis* showed the highest antimicrobial activities against the selected indicator microorganisms, they were selected for further experiments.

3.2. Effect of pH on the antimicrobial activities

Antimicrobial activities of *B. longum* and *L. lactis* subsp. *lactis* at different pH levels (2.0, 4.0 and 6.0) are shown in Table 4. Bacteriocins produced by the selected LAB showed the highest activities at pH 2.0 followed by significant (p < 0.05) decreases at pH 4.0 and 6.0. At pH 8.0, the antimicrobial activities were completely lost (data not shown). Similar results were recorded by Rabie et al. [42] and Bazaraa et al. [15]. Joshi et al. [43] reported that the maximum bacteriocin activities were achieved at pH values below 5.0. Similarly, pH range of 2.0-3.0 was necessary to reach the highest bacteriocin activities followed by a dramatic decrease at higher pH values [44]. Adesina and Enerijiofi [45] reported the highest bacteriocin activities at the acidic pH range of 2.0 to 5.0. Therefore, the use of bacteriocins in acidic and acidified foods is recommended to extend food shelf life.

3.3. Mutagenesis

The resistance of *L. lactis* subsp. *lactis* and *B. longum* against preloaded various antibiotic discs was evaluated. Results revealed high sensitivity of *L. lactis* subsp. *lactis* against 300 µg rifampicin (27 mm) followed by chloramphenicol (20 mm) and doxycycline (20 mm)), while the strain was resistant against other antibiotics (Table 5). In contrast, *B. longum* was only resistant against amoxicillin and sensitive to other antibiotics especially gentamycin (25 mm) followed by chloramphenicol (20 mm) and doxycycline (15 mm). Therefore, such antibiotics were used as markers to select the most resistant mutants.

Table 4. Antagonistic activity of lactic acid bacteria at various pH levels (2, 4 and 6)

Indicator microorganisms	*L. lactis* subsp. *lactis*			*Bifidobacterium longum*		
	2	4	6	2	4	6
Staphylococcus aureus	*5.5^{d}_{c}±0.05	2.7^{c}_{d}±0.05	2.3^{c}_{d}±0.05	10.3^{a}_{a}±0.05	7.0^{a}_{b} ±0.08	6.3^{a}_{bc}±0.05
Salmonella typhimurium	8.6^{b}_{a}±0.05	5.6^{b}_{b} ±0.05	5.0^{b}_{b}±0.05	8.3^{b}_{a}±0.05	5.0^{b}_{b} ±0.05	3.6^{b}_{c}±0.05
Bacillus cereus	7.8^{c}_{a}±0.05	6.6^{ab}_{bc}±0.05	4.0^{b}_{d}±0.08	8.3^{b}_{a}±0.05	6.6^{ab}_{bc}±0.05	6.0^{a}_{c}±0.08
Bacillus subtilis	3.5^{e}_{c}±0.05	3.0^{c}_{c} ±0.05	1.6^{c}_{d}±0.08	7.6^{c}_{a}±0.05	5.6^{b}_{b} ±0.05	3.6^{b}_{c}±0.05
Escherichia coli	11.0^{a}_{a}±0.05	7.6^{a}_{b}±0.05	7.0^{a}_{b}±0.08	7.3^{d}_{b}±0.05	6.3^{ab}_{bc}±0.05	5.3^{a}_{c}±0.05
Klebsiella pneumoniae	5.3^{d}_{a}±0.00	3.6^{c}_{b}±0.05	2.3^{c}_{c}±0.05	5.4^{f}_{a}±0.05	4.0^{c}_{b} ±0.00	2.3^{c}_{c}±0.05
Listeria monocytogenes	10.5^{a}_{a}±0.05	6.0^{b}_{b}±0.05	5.0^{b}_{c}±0.05	6.6^{e}_{b}±0.05	4.3^{c}_{c} ±0.05	3.3^{bc}_{d}±0.05

*Means activity (mm) followed by different superscripts (within columns) and different subscripts (within rows) are significantly different (p<0.05).

Table 5. Antibiotic susceptibility of the selected bacteriocins hyper-producing lactic acid bacteria

Strain	Antibiotic disc									
	*AMX	C	GEN	CT	RI	PB	S	SXT	DOX	TOB
	+25	10	10	25	5	300	10	500	30	10
L. lactis subsp. *lactis*	R	**20	R	R	27	R	R	R	20	R
Bifidobacterium longum	R	20	25	11	10	10	12	10	15	7

*AMX, amoxycillin; C, chloramphenicol; GEN, gentamycin; CT, colistin; RI, rifampicin; PB, polymyxin B; S, streptomycin; SXT, sulphamethoxazole; DOX, doxycycline; TOB, tobramycin.+ Concentration (µg/disc). R= resisted (no clear zone),

**Means of diameter of inhibition zones (mm).

3.4. EMS mutagenesis

In general, 15 antibiotic resistant mutants were selected from each strain and their antimicrobial activities were assessed (Tables 6 and 7). Table 6 shows the antimicrobial activities of *L. lactis* subsp. *lactis* mutants against indicator microorganisms. All the 15 mutants (LM 1 to LM 15) demonestrated higher (p<0.05) activities against *Staphylococcus aureus* and *Bacillus subtilis* when compared to those the parental strains did. On the other hand, mutants showed varied results on the rest of indicator microorganisms. For example, only 26.7% of the mutants showed higher activities against *Listeria monocytogenes*, while 26.6, 86.7 and 86.7% of the mutants were of higher activities against *E. coli*, *Salmonella typhimurium*, and *Bacillus cereus*, respectively. Mutant LM13 recorded the highest antimicrobial activities against all indicator microorganisms followed by LM6. The improvement range for LM 13 was from 39.5 (*Salmonella typhimurium*) to 317.1% (*Bacillus subtilis*). While it was from 9.1 (*E. coli*) to 271.4% (*Bacillus subtilis*) for the mutant LM 6. Table 7 represents antimicrobial activities of the *B. longum* mutants against indicator microorganisms. All mutants showed various responses; for example only 20.0 and 33.3% of the mutants were of higher antimicrobial effects against *Staphylococcus aureus* and *E. coli*, respectively compared to those of the parental strains.

Table 6. Antagonistic activity of *Lactobacillus lactis* subsp. *lactis* mutants

Mutants	Indicator microorganisms						
	Staphylococcus aureus	*Salmonella typhimurium*	*Bacillus cereus*	*Bacillus subtilis*	*Escherichia coli*	*Klebsiella pneumoniae*	*Listeria monocytogenes*
	Inhibition zone (mm ± SD)						
Parent	*5.5$^{h}_{d}$± 0.05	8.6$^{e}_{b}$ ± 0.00	7.8$^{f}_{c}$± 0.05	3.5$^{g}_{e}$ ± 0.00	11.0$^{e}_{a}$ ± 0.05	5.3$^{e}_{d}$ ± 0.00	10.5$^{d}_{a}$± 0.00
LM1	7.3$^{ef}_{c}$± 0.05	10.0$^{cd}_{b}$ ± 0.00	10.6$^{e}_{b}$± 0.05	11.3$^{cde}_{a}$±0.05	10.0$^{f}_{b}$ ± 0.00	7.0$^{c}_{c}$ ± 0.00	10.6$^{d}_{b}$± 0.05
LM2	8.0$^{e}_{de}$±0.00	10.0$^{cd}_{ab}$ ±0.00	10.6$^{e}_{a}$± 0.05	10.6$^{ef}_{a}$ ± 0.05	9.3$^{g}_{bc}$±0.05	9.0$^{b}_{cd}$±0.00	9.6$^{e}_{bc}$±0.08
LM3	7.5$^{ef}_{d}$± 0.05	10.0$^{cd}_{ab}$± 0.00	10.6$^{e}_{a}$± 0.05	10.3$^{ef}_{a}$± 0.05	7.6$^{h}_{d}$± 0.05	9.0$^{b}_{c}$ ± 0.00	9.3$^{e}_{bc}$±0.05
LM4	7.0$^{f}_{e}$ ± 0.00	10.6$^{bc}_{b}$± 0.05	9.0$^{f}_{c}$± 0.05	11.6$^{cde}_{a}$±0.05	7.0$^{i}_{e}$ ± 0.00	6.3$^{d}_{e}$ ± 0.05	8.0$^{f}_{d}$ ±0.00
LM5	6.0$^{g}_{d}$ ± 0.00	12.0^{a} $_{a}$ ± 0.00	12.6$^{cd}_{a}$±0.00	11.0$^{def}_{b}$±0.00	10.6$^{f}_{b}$± 0.05	7.6$^{c}_{c}$ ± 0.05	5.0$^{g}_{e}$ ±0.00
LM6	11.0$^{b}_{d}$ ± 0.00	12.3$^{a}_{bc}$ ± 0.05	13.3$^{bc}_{a}$±0.05	13.0$^{b}_{ab}$±0.00	12.0^{d} $_{c}$±0.00	9.0$^{b}_{e}$ ± 0.00	10.6$^{d}_{d}$ ±0.05
LM7	12.0$^{a}_{a}$ ± 0.00	10.0$^{cd}_{c}$± 0.00	12.6$^{cd}_{a}$± 0.05	10.3$^{ef}_{bc}$±0.07	11.0$^{e}_{b}$ ±0.00	9.0$^{b}_{d}$ ± 0.00	10.6$^{d}_{bc}$±0.05
LM8	9.0$^{d}_{cd}$±0.00	8.6$^{e}_{d}$ ± 0.05	10.6$^{e}_{b}$ ± 0.05	12.0$^{bcd}_{a}$±0.05	9.0$^{g}_{cd}$± 0.00	7.0$^{c}_{e}$ ± 0.00	9.6$^{e}_{c}$ ±0.05
LM9	10.0$^{c}_{e}$± 0.00	11.6$^{ab}_{bc}$±0.06	12.0$^{d}_{ab}$±0.00	12.6$^{bc}_{a}$± 0.05	11.0$^{e}_{cd}$±0.00	10.0$^{a}_{e}$ ±0.00	10.6$^{d}_{de}$±0.05
LM10	12.0$^{a}_{ab}$±0.00	12.6$^{a}_{a}$ ± 0.05	12.0$^{d}_{ab}$±0.00	12.6$^{bc}_{a}$± 0.05	4.0$^{j}_{c}$± 0.00	3.0$^{g}_{d}$± 0.00	11.6$^{c}_{b}$ ± 0.05
LM11	10.0$^{c}_{b}$± 0.00	2.6$^{f}_{d}$ ± 0.05	10.6$^{e}_{b}$ ± 0.05	13.0$^{b}_{a}$ ± 0.05	3.0$^{k}_{d}$ ±0.00	4.6$^{f}_{c}$ ± 0.05	10.0$^{de}_{b}$±0.00
LM12	9.0$^{d}_{b}$± 0.00	10.6$^{bc}_{a}$± 0.05	9.0$^{f}_{b}$ ± 0.00	10.0$^{f}_{a}$ ± 0.00	10.0$^{f}_{a}$ ±0.00	6.0$^{d}_{d}$± 0.00	7.6$^{f}_{c}$ ± 0.05
LM13	12.6$^{a}_{d}$ ± 0.05	12.0$^{a}_{d}$ ± 0.00	16.0$^{a}_{b}$± 0.00	14.6$^{a}_{c}$ ± 0.00	18.0$^{a}_{a}$±0.00	10.6$^{a}_{e}$± 0.05	14.6$^{a}_{c}$ ± 0.05
LM14	10.0$^{c}_{d}$± 0.00	10.6$^{bc}_{d}$±0.05	14.0$^{b}_{b}$ ±0.00	11.6$^{cde}_{c}$±0.57	16.0$^{b}_{a}$± 0.00	4.0$^{f}_{e}$± 0.00	13.6$^{b}_{b}$ ±0.05
LM15	12.6$^{a}_{c}$± 0.05	10.0$^{cd}_{d}$ ±0.00	13.0$^{bcd}_{b}$±0.00	10.6$^{ef}_{d}$ ± 0.05	14.0$^{c}_{a}$± 0.05	9.0$^{b}_{e}$± 0.00	13.0$^{b}_{b}$ ±0.00

*Means followed by different superscripts (within columns) and different subscripts (within rows) are significantly different (p<0.05).

Table 7. Antagonistic activity of *Bifidobacterium longum* mutants

Mutants	Indicator microorganisms						
	Staphylococcus aureus	*Salmonella typhimurium*	*Bacillus cereus*	*Bacillus subtilis*	*Escherichia coli*	*Klebsiella pneumoniae*	*Listeria monocytogenes*
	Inhibition zone (mm ± SD)						
Parent	*10.3$^{d}_{a}$±0.01	8.3$^{e}_{b}$±0.01	8.3$^{g}_{b}$±0.01	7.6$^{f}_{c}$ ± 0.01	7.3$^{e}_{c}$± 0.01	5.4$^{f}_{e}$±0.01	6.6$^{f}_{d}$±0.01
BM1	6.3$^{g}_{c}$±0.05	10.6$^{d}_{a}$±0.05	10.3$^{c}_{a}$±0.09	10.6$^{d}_{a}$ ± 0.05	10.6$^{c}_{a}$±0.09	5.3$^{f}_{d}$±0.05	7.3$^{f}_{b}$±0.05
BM2	10.3$^{d}_{b}$±0.05	10.3$^{d}_{b}$±0.05	12.6$^{c}_{a}$±0.05	10.6$^{d}_{b}$ ± 0.05	10.3$^{c}_{b}$±0.05	9.3$^{b}_{c}$±0.05	10.3$^{c}_{b}$±0.05
BM3	10.6$^{d}_{b}$±0.05	10.3$^{d}_{b}$±0.05	7.3$^{h}_{d}$±0.05	10.6$^{d}_{b}$ ± 0.09	6.3$^{f}_{e}$±0.05	8.3$^{c}_{c}$±0.05	11.3$^{b}_{a}$±0.06
BM4	14.6$^{b}_{a}$±0.05	16.3$^{a}_{a}$±0.05	14.6$^{b}_{b}$±0.05	13.6$^{a}_{c}$ ± 0.08	16.3$^{a}_{a}$±0.05	10.6$^{a}_{d}$±0.08	15.3$^{a}_{b}$±0.05
BM5	10.3$^{d}_{a}$±0.05	8.3$^{e}_{d}$±0.05	11.3$^{d}_{a}$±0.05	10.3$^{d}_{b}$ ± 0.05	8.3$^{d}_{d}$±0.05	9.3$^{b}_{c}$±0.05	8.3$^{e}_{d}$±0.05
BM6	12.0$^{c}_{a}$±0.05	11.3$^{c}_{b}$±0.08	7.6$^{h}_{e}$ ±0.09	11.3$^{c}_{b}$ ± 0.05	5.6$^{g}_{f}$±0.09	10.6$^{a}_{c}$±0.09	9.6$^{d}_{d}$±0.08
BM7	9.3$^{e}_{a}$±0.05	2.3$^{h}_{d}$±0.05	2.3$^{l}_{d}$ ±0.05	4.3$^{h}_{b}$ ± 0.05	2.3$^{j}_{d}$±0.05	3.3$^{h}_{c}$±0.05	3.3$^{i}_{c}$ ±0.05
BM8	7.3$^{f}_{a}$±0.05	1.6$^{i}_{e}$ ±0.05	3.3$^{k}_{c}$±0.05	4.3$^{h}_{b}$ ± 0.05	2.3$^{j}_{d}$±0.05	2.6$^{i}_{d}$±0.09	4.3$^{i}_{b}$ ±0.05
BM9	7.6$^{f}_{a}$±0.05	3.3$^{g}_{d}$±0.05	5.6$^{i}_{b}$ ±0.08	7.3$^{g}_{a}$ ± 0.05	7.6$^{e}_{a}$±0.09	5.6$^{ef}_{b}$±0.05	4.6$^{i}_{c}$ ±0.08
BM10	5.3$^{h}_{b}$±0.05	5.6$^{f}_{b}$±0.08	4.6$^{j}_{c}$ ±0.09	3.3$^{i}_{d}$ ± 0.05	6.6$^{f}_{a}$±0.05	4.6$^{g}_{c}$±0.08	6.0$^{g}_{a}$ ±0.05
BM11	4.3$^{i}_{a}$± 0.05	3.3$^{g}_{b}$±0.05	2.3$^{l}_{c}$ ±0.05	2.6$^{j}_{c}$ ±0.05	3.3$^{i}_{b}$±0.05	3.6$^{h}_{b}$±0.05	3.6$^{j}_{b}$ ±0.08
BM12	6.3$^{g}_{f}$± 0.05	13.6$^{b}_{b}$±0.08	15.3$^{a}_{a}$±0.05	12.6$^{b}_{c}$ ±0.05	12.6$^{b}_{c}$±0.05	9.3$^{b}_{e}$±0.05	10.3$^{c}_{d}$±0.05
BM13	4.3$^{i}_{f}$ ±0.05	13.3$^{b}_{b}$±0.05	14.6$^{b}_{a}$±0.05	13.0$^{a}_{b}$± 0.05	5.3$^{g}_{e}$±0.05	7.3$^{d}_{d}$±0.05	9.3$^{d}_{c}$±0.05
BM14	10.0$^{d}_{a}$±0.05	2.3$^{h}_{f}$± 0.05	9.3$^{f}_{b}$±0.05	8.6$^{e}_{c}$ ± 0.05	4.3$^{h}_{e}$±0.05	8.3$^{c}_{c}$± 0.05	5.3$^{h}_{d}$±0.05
BM15	18.0$^{a}_{a}$±0.16	2.3$^{h}_{d}$± 0.05	10.0$^{e}_{b}$±0.05	10.3$^{d}_{b}$± 0.05	3.3$^{i}_{d}$ ±0.05	5.3$^{f}_{c}$±0.05	6.0$^{g}_{c}$±0.05

*Means followed by different superscripts (within columns) and different subscripts (within rows) are significantly different (p<0.05)

In contrast, 46.7% of the mutants showed antimicrobial activity improvements against and *Salmonella typhimurium*, *Bacillus cereus* and *Listeria monocytegenes*. Also, 53.3 and 66.7% of the mutants included higher activities against *Klebsiella pneumoniae* and *Bacillus subtilis*, respectively, compared to those of the parental strains. Mutant BM4 represented the highest antimicrobial activities against all indicator microorganisms ranged from 41.8 (*S. aureus*) to 131.8% (*Listeria monocytogenes*).

Mutagenesis of LAB strains is widely used for the improvement of LA production using various mutagens [23,26,27]. Bacteriocins production from *L. plantarum* was enhanced utilizing UV, followed by NTG and an improvments of 290 [46] and 266% [47] were achieved. Deletion of the gene encoding Abrb protein resulted in an 84-fold increase in subtilosin by *Bacillus subtilis* when cultured under oxygen-limiting conditions compared to under aerobic conditions [48].

3.5. Intergeneric protoplast fusion

The best hyper-producing mutants LM 6, LM 13, BM 4, and BM 12 were selected for protoplast fusion. First, the mutants were assessed for their antibiotic susceptibility responses and data indicated that, LM 6 and LM 13 were resistant to kanamycin, streptomycin and tobramycin (Table 8). Additionally, LM 13 was resistant to lincomycin. Mutant BM 4 was resistant to kanamycin, rifampicin, streptomycin and tobramycin. Mutant BM 12 was resistant to the same antibiotics except for rifampicin. Based on the antibiotic response patterns, one cross between LM 13 and BM 4 was suggested to carry out the intergeneric protoplast fusion. In further enhancing antimicrobial activities, two parental strains of: *L. lactis* subsp. *lactis* LM 13 and *B. longum* BM 4 were used and fusants resisted lincomycin and rifampicin were selected. The fusants were assessed for their antimicrobial activity (Table 9).

Table 8. Antibiotic susceptibility of the selected bacteriocins hyper-producing mutants

Mutant	Antibiotic disc							
	*AMP	C	ERY	K	L	RI	S	TOB
	+10	30	15	30	2	30	10	30
LM6	16	**21	20	R	8	12	R	R
LM13	15	23	21	R	R	19	R	R
BM4	20	25	23	R	12	R	R	R
BM12	18	25	20	R	12	20	R	R

*AMP, ampicillin; C, chloramphenicol; ERY, erythromycin; K, kanamycin; L, lincomycin; RI, rifampicin; S, streptomycin; TOB, tobramycin. + Concentration (µg/disc). R= resisted (no clear zone).
** Means of diameter of inhibition zones.

Totally, 12 fusants were obtained, however, only F1 fusant exhibited higher (p<0.05) antagonistic effects against all indicator microorganisms, compared to those of the parental strains LM 13 and BM 4 did. The improvement range was from 13.7 (*Listeria monocytegenes*) to 44.2 % (*Salmonella typhimurium*). After a successful mutation followed by protoplast fusion, increased range of 58.1 (*Listeria monocytogenes*) to 345.7% (*Bacillus subtilis*) was achieved compared to the parental strain of *L. lactis* subsp. *lactis*. Increased range of 51.5 (*Staphylococcus aureus*) to 168.5% (*E. coli*) was also obtained compared to *B. longum* parental strain.

Table 9. Antagonistic activity of the parental strains (LM 13 and BM 4) and their fusants.

Fusants	Indicator microorganisms						
	Staphylococcus aureus	*Salmonella typhimurium*	*Bacillus cereus*	*Bacillus subtilis*	*Escherichia coli*	*Klebsiella pneumoniae*	*Listeria monocytogenes*
	Inhibition zone (mm ± SD)						
LM13	*12.6$^{c}_{d}$ ±0.05	12.0$^{c}_{d}$± 0.00	16.0$^{a}_{b}$ ± 0.05	14.6$^{ab}_{c}$±0.05	18.0$^{b}_{a}$±0.00	10.6$^{b}_{e}$ ± 0.05	14.6$^{ab}_{c}$ ±0.05
BM4	14.6$^{b}_{cd}$± 0.81	16.3$^{b}_{a}$± 0.00	14.6$^{b}_{bc}$±0.05	13.6$^{b}_{d}$±0.05	16.3$^{c}_{a}$±0.00	10.6$^{b}_{e}$ ± 0.05	15.3$^{ab}_{b}$ ±0.00
F1	15.6$^{a}_{c}$ ± 0.05	17.3$^{a}_{b}$ ± 0.05	17.3$^{a}_{b}$± 0.05	15.6$^{a}_{c}$± 0.05	19.6$^{a}_{a}$± 0.05	12.6$^{a}_{d}$ ± 0.05	16.6$^{a}_{bc}$ ±0.09
F2	5.3$^{fg}_{d}$± 0.05	8.6$^{d}_{abc}$±0.05	5.6$^{e}_{d}$ ± 0.05	8.3$^{e}_{bc}$±0.05	7.6$^{gh}_{c}$±0.05	9.3$^{cd}_{ab}$±0.05	9.6$^{ef}_{a}$ ± 0.05
F3	7.6$^{e}_{c}$ ± 0.05	3.6$^{h}_{d}$ ± 0.05	1.1$^{c}_{a}$ ± 0.09	11.6$^{c}_{a}$± 0.05	9.3$^{f}_{b}$± 0.05	10.0 $^{bc}_{b}$±0.00	12.3$^{cd}_{a}$± 0.05
F4	4.3$^{g}_{d}$± 0.05	8.6$^{d}_{b}$ ±0.05	5.0$^{e}_{d}$ ± 0.08	5.0$^{f}_{d}$ ±0.00	8.0$^{g}_{bc}$±0.05	7.3$^{e}_{c}$ ± 0.05	13.6$^{bc}_{a}$± 0.05
F5	13.3$^{bc}_{a}$±0.05	12.6$^{c}_{a}$ ± 0.05	12.3$^{c}_{ab}$± 0.05	8.6$^{de}_{d}$±0.05	11.0$^{e}_{c}$± 0.08	8.6$^{d}_{d}$ ± 0.05	11.3$^{de}_{bc}$±0.05
F6	10.6$^{d}_{c}$ ± 0.05	12.0$^{c}_{b}$ ± 0.08	12.3$^{c}_{b}$ ± 0.05	9.6$^{d}_{c}$ ±0.05	12.6$^{d}_{ab}$±0.05	9.6$^{bcd}_{c}$±0.05	13.6$^{bc}_{a}$±0.05
F7	6.3$^{f}_{b}$ ± 0.05	5.6$^{f}_{b}$± 0.05	2.6$^{f}_{c}$ ± 0.05	3.0$^{h}_{c}$ ±0.08	5.6$^{ij}_{b}$± 0.05	3.6$^{fg}_{c}$ ±0.05	12.3$^{cd}_{a}$±0.05
F8	5.3$^{fg}_{b}$± 0.05	4.6$^{g}_{b}$ ± 0.05	2.6$^{f}_{c}$ ± 0.05	3.6$^{gh}_{bc}$±0.07	5.3$^{j}_{b}$ ± 0.05	4.6$^{f}_{b}$ ± 0.05	8.0$^{fg}_{a}$±0.22
F9	9.6$^{d}_{a}$ ± 0.05	7.3$^{e}_{b}$± 0.05	5.6$^{e}_{bc}$±0.05	4.3$^{fg}_{cd}$±0.05	3.3$^{k}_{d}$± 0.12	3.3$^{g}_{d}$± 0.05	7.0$^{gh}_{b}$±0.22
F10	5.6$^{f}_{cd}$ ±0.05	7.3$^{e}_{b}$ ± 0.00	9.6$^{d}_{a}$ ±0.05	5.3$^{f}_{d}$ ± 0.05	8.6$^{fg}_{a}$± 0.05	6.6$^{e}_{bc}$±0.05	8.6$^{fg}_{a}$±0.02
F11	9.6$^{d}_{a}$ ± 0.05	9.0$^{d}_{a}$ ± 0.05	6.0$^{e}_{bc}$±0.08	3.0$^{h}_{d}$ ±0.08	6.6$^{hi}_{b}$± 0.05	4.6$^{f}_{cd}$±0.12	6.6$^{gh}_{b}$±0.12
F12	10.6$^{d}_{a}$ ± 0.05	4.6$^{g}_{bc}$±0.05	5.6$^{e}_{b}$ ±0.05	5.0$^{f}_{b}$ ± 0.08	5.3$^{j}_{b}$± 0.05	3.6$^{fg}_{c}$±0.05	5.0$^{h}_{b}$± 0.08

*Means followed by different superscripts (within columns) and different subscripts (within rows) are significantly different (p<0.05).

Zhang et al. [49] improved nisin production of *L. lactis* subsp. *lactis* from 500 to 14000 IU ml^{-1} using UV-irradiation followed by four rounds of protoplast fusion. Nitrosoguanidine followed by protoplast fusion was applied by Dahikar and Bhutada [30] to enhance LA production from *L. rhamnosus* with only a slight increase (83.6%).

4. Conclusion

In conclusion, EMS mutagenesis followed by intergeneric protoplast fusion successfully resulted in obtaining four hyper-bacteriocins producing mutants as well as one fusant. Such mutants and fusant can include a great economic values.

5. Conflict of Interest

The authors report no conflicts of interest.

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جهش‌زایی و ادغام پیش‌یاخته به‌منظور افزایش تولید باکتریوسین‌ها

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چکیده

سابقه و هدف: جهش‌زایی القایی به‌طور گسترده برای تولید جهش‌های جدید با بازدهی بهبود یافته مورد استفاده قرار می‌گیرد. به‌منظور بهبود گونه‌های باکتری‌های لاکتیک اسید دارای فعالیت بالای ضدمیکروبی، جهش‌زایی القایی با اتیل متان سولفونات به‌دنبال ادغام اینترژنریک پیش‌یاخته انجام شد.

مواد و روش ها: فعالیت‌های آنتاگونیستی هفت گونه باکتری‌های لاکتیک اسید در برابر هفت میکروارگانیسم شناساگر به‌روش انتشار چاهک بررسی شد. دو گونه با بیشترین اثر با اتیل متان به‌دنبال ادغام اینترژنریک پیش-یاخته، جهش‌زایی شدند. انتخاب جهش یافته‌ها و ادغام کننده‌ها بر اساس پاسخ به آنتی‌بیوتیک‌های گوناگون بوده است.

یافته‌ها و نتیجه‌گیری: *لاکتوباسیلوس لاکتیس* زیرگونه *لاکتیس* و *بیفیدوباکتریوم لانگام* بیشترین فعالیت ضدمیکروبی در برابر اغلب میکروارگانیسم‌های شناساگر را داشتند. چنین فعالیتی در pH برابر ۲ حاصل شد و با افزایش pH کاهش چشمگیری داشت. با جهش‌های القایی اتیل متان سولفونات سی جهش‌یافته به‌دست آمد، که سه تا از آنها فعالیت بیشتری در مقایسه با انواع گونه‌های والد (دو تا برای هر والد) داشتند. جهت افزایش این فعالیت، ادغام اینترژنریک پیش‌یاخته‌های LM 13 (حاصل *لاکتوباسیلوس لاکتیس* زیرگونه *لاکتیس*) و BM 4 (حاصل *بیفیدوباکتریوم لانگام*) انجام شد و۱۲ ادغام یافته به‌دست آمد. جالب اینکه، در یک ادغام یافته (F1) فعالیت ضدمیکروبی در مقایسه با گونه‌های والدش افزایش داشت. افزایش ۵۸/۱ تا ۳۴۵/۷ درصدی در مقایسه با گونه والد *لاکتوباسیلوس لاکتیس* زیرگونه *لاکتیس* و ۵۱/۵ تا ۱۶۸/۵ درصدی برای دومین گونه والد مشاهده شد.جهش‌یافته-های LM 13، LM 6، BM4 و BM12 و ادغام یافته F1 می توانند در نگهداری فرآورده‌های غذایی مورد استفاده قرارگیرند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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- باکتریوسین‌ها
- *بیفیدوباکتریوم لانگام*
- اتیل متان سولفونات
- ادغام پیش‌یاخته اینترژنریک
- *لاکتوباسیلوس لاکتیس* زیرگونه *لاکتیس*

***نویسنده مسئول**

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