JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2013. Vol. **7**(4), p. 2673-2689

A Complementary "*In Vitro*" Study of Bacteriocinogenic Activity and Probiotic Characteristics of Newly Isolated *Enterococcus faecium* SFD

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(Received: 29 September 2013; accepted: 08 November 2013)

In an attempt to search for candidate beneficial probiotic strain of *Enterococcus faecium*, isolate recovered from traditional Egyptian cheese (karish) was identified and evaluated regarding their antimicrobial activity and their standard biochemical profile. Bacteriocin SFD is highly inactivated by Papain, Trypsin, EDTA (0.5mM), DTT and Triton X-100, but not when treated with pH values between 2.0 and 8.0 for one hour, and after treatment at 40ºC for 15 min. Activity was, however, lost after treatment at 100ºC for 30 min. The highest level of activity (25600 AU/ml-1) was recorded when cells were grown in MRS broth, pH 6.5 after 20 h. Bacteriocin SFD differs from other broad-spectrum bacteriocins described for *Enterococcus* spp. by being extremely active against Gram-negative bacteria and by being smaller. The newly isolated culture was evaluated for a number of probiotic characteristics like viability under gut-like conditions, cell surface hydrophobicity, autoaggregation, coaggregation, phenol tolerance, adhesion to intestinal mucousand cholesterol assimilation. *In vitro* results obtained showed that the isolated *Enterococcus faecium* SFD, was able to meet the basic requirements for probiotic functions as they demonstrated probiotic characteristics such as tolerance to pH 2.5, growth in 0.5% bile salts, growth in 0.3% phenol, hydrophobicity of 37%, adhesion to intestinal mucous and 35% cholesterol assimilation ability. In the light of this study, it is observed that, *Enterococcus faecium* SFD is regarded as a promising candidate probiotic and adjunct culture for new healthy food product.

Key words: Probiotics, Lactic acid bacteria, Bile resistance, Acid tolerance, Adhesion, Bacteriocin.

Recently, a great deal of scientific focus has moved the primary role of food as the source of essential nutrient required as energy and body-forming substances to the more subtle action of biologically active food components on human health. There has been an explosion of health-conscious consumers interest in the active role of food in the well-being and life prolongation, as well as in the prevention of initiation, promotion, and development of nontransmissible chronic diseases. As a result, a new term- functional food- was proposed. Among these foods, probiotics may exert positive effects on the composition of gut microbiota and overall health, and the ability of marketers to create new interest in existing products[1,2].

The beneficial effects of food with added live microbes (probiotics) on human health are

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being increasingly promoted by health professionals. It has been reported that these probiotics can play an important role in relieving diarrhea, improving lactose intolerance and its immunomodulatory, anticarcinogenic, antidiabetic, hypocholesterolemic, hypotensive properties and could have a significant effect in alleviating infectious disease[3,4]. Among these microorganisms, lactic acid bacteria are regarded as a major group of probiotic bacteria. LAB are a large group of non-pathogenic fermentative, anaerobe facultative, aerotolerant microorganisms which technologically suitable for industrial processes, acid fast; bile tolerant, adhere to the gut epithelial tissue and produce antimicrobial substances, including, organic acids, hydrogen peroxide and bacteriocins (biologically active proteins)[5].

The production of bacteriocins is another property of interest. Lactic acid bacteria are well known for their production of ribosomally synthesized antimicrobial proteins or peptides, collectively known as bacteriocins.

In general, *in vitro* data alone is not sufficient to describe LAB strains as probiotics, but they still remain valuable and can provide scientific insight into characteristics of probiotic organisms. Probiotic strains of the future will be probably selected by means of *in vitro* assays recommended by FAO/WHO[6], but *in vivo* assessment will remain mandatory for a final selection step[7]. Nevertheless, such *in vitro* systems remain powerful tools especially for screening numerous samples. These tests are important since probiotics consist of a wide variety of species and sub-species and the ability to adhere, colonize and modulate the human gastrointestinal system is not a universal property. With this knowledge, optimal probiotic strains may be developed for commercial use[8].

As there is a permanent need to select new beneficial strains with improved capacities in healthy food product, our interest is focused on *Enterococcus* strain that is mainly linked to dairy products. Bacteria of the genus *Enterococcus* are ubiquitous Gram-positive, catalase-negative cocci that often occur and have been used in many different applications as starters or adjunct cultures, and in foods they seems to have a major role in improving flavor development and quality of cheese with some useful biotechnological traits, such as the production of bacteriocins with anti-pathogenic activity[9]. It is well known that *Enterococci* are able to produce various bacteriocins some of them can display fairly broad inhibitory spectra to food-borne pathogenic bacteria

In our study, *Enterococcus faecium* isolate recovered from local dairy products were screened and evaluated according to their antimicrobial activity against some *Salmonella enteritica, Yersinia enterocolitica* and *Bacillus cereus* known for their pathogenicity in humans. Our goal was to investigate the capacity of this enterococcal strain to colonize the intestine while exhibiting probiotic activity including *in vitro* cholesterol-reducing effect.

MATERIALS AND METHODS

Isolation and Identification of Bacteriocin producing strain

Strain of *E. faecium* SFD was isolated from Egyptian karish cheese coming from the Alexandria area, North Egypt, during the year 2010. It was routinely growth in MRS agar (Oxoid, Milan, Italy) at 37ºC for 18–24 h. The spectrum of antimicrobial activity was determined by screening against the strains listed in Table 1. It was identified according to the procedures described in the Bergey's Manual of Systematic Bacteriology[10,11] and API 20 for cocci system (biomerieux, France). Further confirmation of the strain identity was done by sequencing the invariant region in the 16S rDNA sequences for LAB according to Wang *et al.,*[12] obtained by polymerase chain reaction (PCR) using primers (5/ CATCCAGTGCAAACCTAAGAG 3/ and 5/ GATCCGCTTGCCTTCGCA 3/) forward and reverse primers, respectively.

Bacteriocin bioassay

Antimicrobial activity was confirmed by using the agar spot-on-lawn test method, as described by Kwon *et al*.,[13]. The pH of the supernatants was adjusted to 6.0 with sterile 1 N NaOH before testing. Activity was quantified by the critical two fold dilution method, currently used for the assay of bacteriocins[14,15]. Activity was expressed as arbitrary units (AU ml^{-1}). One AU was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition. The indicator strains are listed in Table 1.

Study of growth pattern and bacteriocin production

An 18 h old culture of strain was inoculated (2%, v/v) into MRS broth followed by incubation at 37ºC, without agitation, for 24 h. Samples were taken at 2 hours intervals during 24 h. The growth was followed by measuring optical density (OD) at 620 nm. Bacteriocin activity against *Salmonella enterica* ATCC 25566 (AU/ml^{-1}) was expressed in AU ml^{-1}, as described by Parente *et al*.,[14]. The experiment was performed in triplicates.

Sensitivity of bacteriocin to heat, pH and proteolytic enzymes

The bacteriocin activity of the cell free culture supernatant of the selected isolated strain was analysed after heat-treatment, treatment with proteolytic enzymes, and at different pH values. The residual bacteriocin activity was determined by agar spot test. The bacteriocin sample was heated[16], at 40, 60, 80 and 100ºC for 15, 30 and 60 min before the activity test. To test the sensitivity to proteases, aliquots of 1ml bacteriocin preparation were treated with trypsin (Universal Fine Chemical PVT, LTD, India), pepsin (Sigma, St Louis, MO, USA), papin (Titan Biotech, LTD) at a final concentration of 1 mg/ml at 37ºC for 2 h. Cell free culture supernatant was also treated with catalase (1 mg/ml) in order to completely eliminate possible inhibitory activity due to hydrogen peroxide. An untreated culture extract and protease (in buffer only) served as controls. To test the influence of pH, the cell free culture supernatant was adjusted to various pH values ranging from 2.0 to 10.0 (at increments of one pH units) using either HCl or NaOH, and incubated for 1h at 37ºC. The pH-treated samples were neutralized to pH 6.0 before measuring the residual bacteriocin activity. In this case, MRS broth (pH range 2–10), served as a control[17]. The residual bacteriocin activity (AU/ml^{-1}) was measured by bioassay, using *Bacillus cereus* ATCC 49064, *Salmonella enterica* ATCC 25566 as indicator strains.

Effect of detergents on bacteriocin activity

Cell free supernatant of the selected isolate were treated with the following detergents: Tween 20, Tween 80, Triton X-100, Triton X-114, sodium-dodecyl sulphate (SDS), β-mercaptoethanol, urea, dithiothreitol (DTT), at final concentrations of 1%, or by EDTA at a final concentration of 0.1 , 0.3 and 0.5 mM. The control consisted of either cell free supernatant or detergent, in 50 mM sodium phosphate buffer, pH 7.0. All samples and controls were incubated at 37ºC for 5 h[18], and titers of bacteriocin activity were determined.

Molecular size of the bacteriocin

Strain of *Enterococcus faecium* SFD was grown in MRS broth for 20 h at 30ºC. The cells were harvested by centrifugation (6500*xg*, 10 min, 4ºC) and the bacteriocin precipitated from the cell-free supernatant with 30, 40, 50, 60 and 70% saturated ammonium sulphate[19]. The precipitate was resuspended in one tenth volume sodium phosphate buffer (pH 6.5), desalted by using a 3500 Da cut-off dialysis membrane (Spectrum Inc., CA, USA) and separated by tricine–SDS–PAGE, as described by Schagger and Von Jagow[20]. A low molecular weight marker with sizes ranging from 3.5 to 200 kDa (BioRad) was used. The gels were fixed and half were stained with Coomassie Blue G-250 (Saarchem, Krugersdorp, South Africa). The position of the active peptide band in the gel was determined by overlaying an unstained gel with cells of *Salmonella enterica* ATCC 25566 embedded in soft MRS agar[21].

Evaluation of Potential Probiotic Properties

Viability under gut-like conditions

Viability was tested in a set of experiments, where *E. faecium* SFD was exposed to conditions simulating those in the stomach (gastric juice) and the small intestine (bile salts). Cells were propagated twice in MRS broth, and centrifuged at 4300×*g*, 10 min. The pellet was washed three times in phosphate-buffered saline (PBS; pH 7.0, 0.85% NaCl). The total viable count was determined at each step, time zero, and at the end of incubation. Tolerance to gastric juice was determined according to Charteris *et al*.,[22]. Simulated gastric juice was prepared fresh daily by suspending pepsin (3 g/l) (P-7000, Sigma-Aldrich, St. Louis, MO) in sterile saline (0.5% w/v) and adjusting the pH to 2.0, 2.5, 3.0 and 3.5 with concentrated HCl. Washed cell pellets were then suspended in (1/10)×cultivation volume in the same buffer, hence obtaining a 10-fold increase in cell density. To 1 ml of the washed cell suspension, 5 ml of simulated gastric juice and 1.5 ml NaCl (0.5 w/v) were added and incubated at 37ºC for 3 h.

Tolerance to bile salts was determined according to Matijasic and Rogelj[23] . Bile

containing MRS broth was prepared by the addition of 0.1, 0.3 and 0.5% (v/v) of ox bile (Sigma-Aldrich, B-8381). The cells from a 100 ml 16-18 h MRS tested culture were collected by centrifugation (3400×*g* 10 min), washed twice in saline (8.5g NaCl/l) and resuspended in 10 ml MRS broth. This suspension was inoculated (1%) into MRS broth lacking or containing bile. After 0, 1, 2 and 3 h of incubation at 37ºC, viable counts on MRS agar plates and optical density of the culture at 625 nm were determined.

Cell Surface Hydrophobicity

The ability of the isolated strains to adhere to hydrocarbons was determined according to the method of Thapa *et al.,*[24], with a few modifications. This method was based on adhesion of cells to xylene droplets. Cultures were grown in 10 ml MRS broth, centrifuged at 6,000 ×*g* for 5 min, and the cell pellet was washed twice with 10 ml of sterile Ringer solution (6% NaCl, 0.0075% KCl, 0.01% $CaCl_2$ and 0.01% $NaHCO_3$) and resuspended in 10 ml of the same buffer. The absorbance at 600 nm was measured. Cell suspension was then mixed with equal volume of xylene and mixed thoroughly by vortexing for 2 min. The two phases were allowed to separate for 30 min, and absorbance at 600 nm of the lower phase was recorded. The percentage of cell surface hydrophobicity was calculated as:

$$\left\{ Hydrophobicity\ (\%) = \frac{OD\ 600\ (initial) - OD\ 600\ (with\ xylene) \times 100}{OD\ 600\ (initial)} \right\}$$

Spectrophotometric autoaggregation assay

Autoaggregation assays were performed according to Ekmekci *et al.*,[25] with certain modifications. Bacteria were grown in aerobic and anaerobic conditions for 16 h at 37ºC with MRS liquid medium. The activated cells were harvested by centrifugation at 10,000 *xg* for 15 min, washed twice in phosphate- buffered saline (PBS) containing (g/l): NaCl, 8; KH_2PO_4, 0.34; and K_2HPO_4, 1.21 (pH 6.0) and resuspended in the same buffer to give a final optical density of 0.60 ± 0.02 at 600 nm as measured by a spectrophotometer (Hitachi, T1800). For aerobic and anaerobic autoaggregation experiments, cell suspensions (2 ml) were mixed by vortexing for 10 s and autoaggregation was determined during 4 h of incubation at 37ºC. The absorbance (A) was measured at 600 nm after 4 h. The percent autoaggregation was expressed as follows:

$$\% \text{ Autoaggregation} = [(A_1 - A_2) / (A_1) \times 100]$$

Where A_1 represents the cell absorbance at zero time and A_2 the cell absorbance after 4 h.

Coaggregation assays

Coaggregation experiments were performed between *E. faecium* SFD strain with other LAB and some pathogenic bacteria (Table 6) according to the method of Basson *et al.,*[26]. The percent coaggregation was expressed as follows:

$$\text{Coaggregation} = (A_{Tot} - A_S / A_{Tot}) \cdot 100$$

Where A_{Tot} refers to absorbance immediately after the strains were paired and A_S refers to the absorbance of the supernatant after 60 min of incubation and centrifugation at 300*xg* for 2 min at 20ºC. Experiments were conducted in triplicate on two separate occasions.

Phenol tolerance

Phenol tolerance experiments were performed as described by Aswathy *et al.*,[27] with some modification. The overnight culture of the promising isolate was inoculated (1%) into MRS broth with or without various phenol concentrations (0.1, 0.2, 0.3, 0.4 and 0.5% (v/v) phenol). Bacterial cells in the culture broth were measured by reading the absorbance (A) at 600 nm after 0, 2, 4, 6, 8, 10, 12, and 24 h of incubation at 37ºC. The experiments were repeated two times in duplicate.

***In vitro* adhesion analyses**

Epithelial cells were isolated from the small intestine (segments of freshly collected duodenum) of a healthy Rabbit, and phase-contrast microscopic examination of the adherence of *E. faecium* SFD to epithelial cells was performed essentially as described by Alwan *et al.,*[28].

Cholesterol assimilation

Cholesterol assimilation from MRS broth by *E. faecium* SFD was carried out as described by Rudel and Morris[29]and modified by Mishra and Prasad[30]. Freshly prepared MRS broth (MRS broth containing 0.2% sodium thioglycolate) (Sigma) was supplemented with 0.3% sodium taurocholate (Sigma) as a bile salt. The sodium thioglycolate functioned as an oxygen scavenger. The buffalo serum (cholesterol sources), was obtained from the slaughterhouse of farm Apis, Animal Production Department, Alexandria university, Egypt. The buffalo serum (having initial total cholesterol content of 110 ± 2.2 dl) was added at a rate of 10% into sterile media and mixed thoroughly.

After incubation (37ºC for 24 h), cells were removed by centrifugation 796 *xg* for 15 min. Then, total cholesterol in the spent broth was measured using the colorimetric method reported by Rudel and Morris[29]. Absorbance was compared to a standard curve prepared by using suitable concentration (1 mg cholesterol / ml ethanol). Uninoculated sterile broth was also analyzed as a negative control.

RESULTS AND DISCUSSION

Selection and identification of bacteriocin-producing strain

Samples from the Karish Egyptian cheese were evaluated for antagonistic isolates with direct antimicrobial activity against selected indicator bacteria (Table 1), and their antimicrobial spectra. From the antagonistic isolates, the chosen strain was identified as a member of the genus *Enterococcus* based on Gram-reaction (positive), absence of catalase, morphology (cocci in pairs) and growth at different temperature, in presence of different NaCl and different pH as described in Bergey's Manual of Determinative of bacteriology[11] for genus *Enterococcus*. Further identification to species level, *E. faecium* SFD, was based on yellow pigment formation on carbohydrate fermentation reactions recorded with API 20 Strep (bioMérieux, Germany). Molecular biological methods were used to verify the identity of this strain. Genotypic characterization, by RAPD–PCR analysis with amplification of 16S rDNA of the selected isolate, was resulted in the synthesis of characteristic single band of about 550 to 600-bp fragment (Fig. 1a). The sequences of the chosen isolate were aligned with the 16S rDNA sequences from the GenBank database to identify the studied microorganism. 16S rDNA sequencing data of the selected isolate clearly showed 76% homology to *E. faecium*. Furthermore, cell morphology of selected strain examined by scanning electron microscopy (SEM) confirmed the normal morphology of *E. faecium* SFD (Fig. 1b). Also Lertworapreecha *et al.,*[31] found this method specific and suitable for genus confirmation. They successfully identified lactic acid bacterial isolates which showed PCR product at 638 bp of 16S rDNA for *E. faecium* with homology of 78.6%.

Evaluation of *E. faecium* SFD isolate for production of bacteriocin

In order to determine the activity of the bacteriocin produced by *E. faecium* SFD strain considered in this study, agar spot-on-lawn method were performed using LAB and non-LAB species as indicator organisms. The results obtained are shown in Table 1. As shown, tested strain did not show any antibacterial activity towards several species of LAB belonging to the genera

Table 1. Antimicrobial activity of a cell-free pH-neutralised supernatant of *E. faecium* SFD

Indicator organism	Temperature (°C)	Medium	Bacteriocin activity
Food spoilage and bathogenic bacteria			
Listeria innocua ATCC 33090	37	BHI	-
Listeria monocytogenes ATCC 19116	37	BHI	-
Listeria ivanovii Li4 (pVS2)a	30	BHI	-
Salmonella enterica ATCC 25566	37	Nutrient Broth	++
Bacillus cereus ATCC 49064	30	Nutrient Broth	++
Yersinia enterocolitica ATCC 23715	37	Tryptose Broth	+
Clostridium perfringens ATCC 13124	37	Trypticase Soya Broth	-
Escherichia coli wild	37	LB Broth	-
Lactic acid bacteria			
Lactococcus lactis subsp. *lactis* ATCC 19435	37	MRS	-
Lactococcus lactis subsp. *lactis* JCM 7638	37	MRS	-
Lactobacillus sakei LTH 673b	37	MRS	-
Lactobacillus sakei CTC 494b	37	MRS	-
Lactobacillus sakei NCDO 2714b	37	MRS	-
Streptococcus thermophilus CCUG 30577	37	MRS	-

[a]kindly supplied Dr. Lars Axelsson (Matforsk, Norwegian Food Research Institute, Norway). [b]kindly provided by Prof. Ingolf Nes (Laboratory of Microbial Gene Technology, Agriculture University of Norway)

Lactobacillus casei, *Lactococcus lactis* subsp. *lactis*, *Lactobacillus sake*, *Lactobacillus plantarum*, nor towards *Streptococcus thermophilus*. Considering the non-LAB, including

Table 2. Effect of various proteolytic enzymes and pHs on antibacterial activity of the cell free supernatant of *E. faecium* SFD against *salmonella enterica* ATCC 49064 and *Bacillus cereus* ATCC 49064

	B. cereus ATCC 49064		S. *enterica* ATCC 25566	
Cell free supernatant treatment	Remaining activity (AU/ml)	Reduction (%)	Remaining activity (AU/ml)	Reduction (%)
Proteolytic enzyme (1mg/ml):				
Papain	0	100	0	100
Pepsin	0	100	200	99.2
Trypsin	200	99.2	800	96.8
Control[2]	0		0	
pH:				
2	25600	0	25600	0
3	25600	0	25600	0
4	25600	0	25600	0
5	25600	0	25600	0
6	25600	0	25600	0
7	25600	0	25600	0
8	800	96.8	800	96.8
9	0	100	0	100
10	0	100	0	100

Table 3. Effect of different heat temperatures on antibacterial activity of cell-free supernatant[1] of *E. faecium* SFD against two pathogenic indicator strains

	B. cereus ATCC 49064		*S. enterica* ATCC 25566	
Temp.	Remaining activity (AU/ml)	Reduction (%)	Remaining activity (AU/ml)	Reduction (%)
40 °C				
15 min	25600	0	25600	0
30 min	3200	87.5	3200	87.5
60 min	3200	87.5	3200	87.5
60 °C				
15 min	3200	87.5	6400	75
30 min	1600	93.75	1600	93.75
60 min	800	96.8	800	96.8
80 °C				
15 min	800	96.8	1600	93.75
30 min	800	96.8	800	96.8
60 min	800	96.8	800	96.8
100 °C				
15 min	800	96.8	1600	93.75
30 min	0	100	0	100
60 min	0	100	0	100

foodborne pathogens and strains technologically relevant for food production, the activity was observed only in the case of *Salmonella enterica*, *Bacillus cereus* and *Yersinia enterocolitica* with strong activity of 25600 AU/ml against *Salmonella enterica* and *Bacillus cereus*.

Growth and production

The relationship between cell growth and bacteriocin production by *E. faecium* SFD as influenced by suboptimal temperature and initial pH values was assessed. Figure 2 represent an example of a fermentation trial at a controlled temperature of 37ºC with an initial pH of 5.5. The antibacterial activity was determined against *Salmonella enterica* ATCC 25566. The culture media of *E. faecium* SFD presented growth curve with a short lag phase of two hours, followed by a logarithmic growth phase of 10 to 12 h (Fig. 2). As

Table 4. Effect of different surfactants on antibacterial activity of cell-free supernatant of *E. faecium* SFD against two pathogenic indicator strains

Surfactants	*B. cereus* ATCC 49064		S. *enterica* ATCC 25566	
	Remaining activity (AU/ml)[1]	Reduction (%)	Remaining activity (AU/ml)[1]	Reduction (%)
Non ionic[2]				
Triton X-100	400	98.4	6400	75
Triton X- 114	1600	93.75	25600	0
Tween 20	1600	93.75	25600	0
Tween 80	1600	93.75	1600	93.75
Anionic				
EDTA (0.1mM)	1600	93.75	25600	0
EDTA (0.3mM)	3200	87.5	25600	0
EDTA (0.5mM)	800	96.8	0	100%
SDS[2]	6400	75	25600	0
Cation[2]				
β-Mercaptoethanol	1600	93.75	1600	93.75
DTT	1600	93.75	0	100
Urea	800	96.8	25600	0

[1]AU: Activity unit.AU: is reciprocal of the highest two-fold dilution that exhibited a clear zone of inhibition [2]At final concentration of 1%

Table 5. Ability of *E. faecium* SFD to survive in gastric juice with various pH values and in presence of various concentrations of bile salts (%) at 37 ºC

Treatments	Mean of viable counts (log_{10} CFU /ml)± SD[1] Time of exposure (h)			
Gastric juice at :	0	1	2	3
PH 6.5 (control)	7.397±0.01	7.380±0.04	7.380±0.007	7.367±0.02
PH 2.5	7.397 ±0.05	7.204±0.004	7.123±0.03	7.123±0.01
PH 2.0	7.397±0.02	0	0	0
Bile salt (%)				
Control	8.06±0.03	8.20±0.009	8.28±0.007	8.41±0.02
0.1	8.06±0.01	8.18±0.01	8.27±0.01	8.27±0.006
0.3	8.06±0.009	8.11±0.05	8.22±0.01	8.26±0.006
0.5	8.07±0.003	8.11±0.04	8.12±0.02	8.12±0.02

[1]Each value in the table represent mean value of three experiments each was carried out in duplicate ± standard deviation (SD). 0: No growth at 10^4 dilutions

previously observed in MRS medium[32,33,34], bacteriocin activity was detected early in the exponential-growth phase after 4 h, followed by continuous production during this phase. The cell density reached the maximum 6 h earlier than bacteriocin production. They reached the maximum biomass concentration with absorbance of 2.4 after 14 h. However, the maximum bacteriocin production of 25600 AU/ml was reached after 20 h of cultivation time (Fig. 2).

Factor affecting antibacterial activity of cell free supernatant of *E. faecium* SFD

Effect of proteolytic enzymes, pH and heat treatment

To verify the protein nature of the inhibitor substance, the cell-free supernatant (CFS) of *E. faecium* SFD was treated by a variety of proteolytic enzymes shown in Table 2.

Complete inactivation of antimicrobial activity against both indicators was observed after treatment of the CFS of tested strain with papen. However, the activity of CFS was completely losted when pepsin was used only with *B. cereus* as indicator strain. The treatment of this CFS with other proteolytic enzymes caused a decrease in its antibacterial activity ranged from 96.8 to 99.2% (Table 2). A similar result was reported with bacteriocin produced by *E. faecium* GM-1 after treatment with proteinase K and papain[35]. These data clearly showed that the antimicrobial substance is of proteinaceous nature, containing cleavage-sites suitable for the mentioned proteases. Based on the obtained results, the antimicrobial agent produced by *E. faecium* SFD was a bacteriocin according to the criteria outlined by Lewus *et al*., and Gonzalez *et al.,*[36,37] and results obtained by Ivanova *et al*., and Pinto *et al*.,[38,39].

The pH stability of the culture supernatant was studied in the range from pH 2 to 10 (Table 2). The activity was not changed under a wide pH range of 2 to 7. However, activity was completely eliminated at pHs of 9 and 10 for 1 h. The exposure to the alkali pH was found to be detrimental to the bacteriocin activity.

Bacteriocins differ greatly with respect to their sensitivity to inactivation by changes in pH. Many of the bacteriocins and bacteriocin-like substances produced by lactic acid bacteria are only stable at acid and neutral pH[40] and are inactivated even at a pH above 8.0 (e.g. nisin, lactostrepcins, pediocin AcH, leucocin A-UAL 187). This can be attributed to the solubility of the bacteriocins of LAB (lactic acid bacteria); the isoelectric points of the bacteriocins produced by LAB are around 8.0-9.0 and the solubility of the bacteriocins decreases with increasing pH.

The stability of the secreted inhibitory compounds was tested using different temperature treatments (Table 3). The original Activity of crude extract (25600 AU/ml) was changed upon all the heat treatment regimes beyond 15 min at 40ºC with complete inactivation after treatment of 100°C for 30 min. Heat resistance is a major characteristic of many bacteriocins and bacteriocin-like compounds produced by lactic acid bacteria and can vary dramatically ranging from 60 to 100ºC for more than 30 min (e.g. lactocin 27, lactocin S, carnobacteriocins A and B) to autoclaving at 121ºC for 15-20 min (e.g. lactacin B, lactacin F, nisin etc.)[40].

Sensitivity of the bacteriocin to detergent agents

To confirm the effect of different detergent on the activity of the cell free supernatant of the tested strain, two indicator strains were used (S. *enterica* ATCC 25566 and *B. cereus* ATCC 49064).

Antagonistic activity of the bacteriocin was greatly reduced after exposure to different tested surfactants when *B. cereus* ATCC 49064

Table 6. *In vitro* characterization of *E. faecium* SFD isolates as potential probiotic

Treatments	Percentage (%) Mean ± SD *E. faecium* SFD
Hydrophobicity	37.1 ± 1.069
Autoaggregation^{aerobic}	14.38 ± 0.797
Autoaggregation^{anerobic}	24.16 ± 0.493
Coaggregation	
Bacillus cereus ATCC 49064	10.8 % ± 0.458
Enterococcus faecium	-
Lactobacillus plantarum	11.4 % ± 0.624
Listeria innocua ATCC 33090	13.6 % ± 0.850
Listeria monocytogenes ATCC 19116	6.11 % ± 0.458
Salmonella enterica ATCC 25566	18.5 % ± 1.135
Adhesion to epithelial cell of rabbit	17.1 ± 3.3
Cholesterol reduction (%)	35%

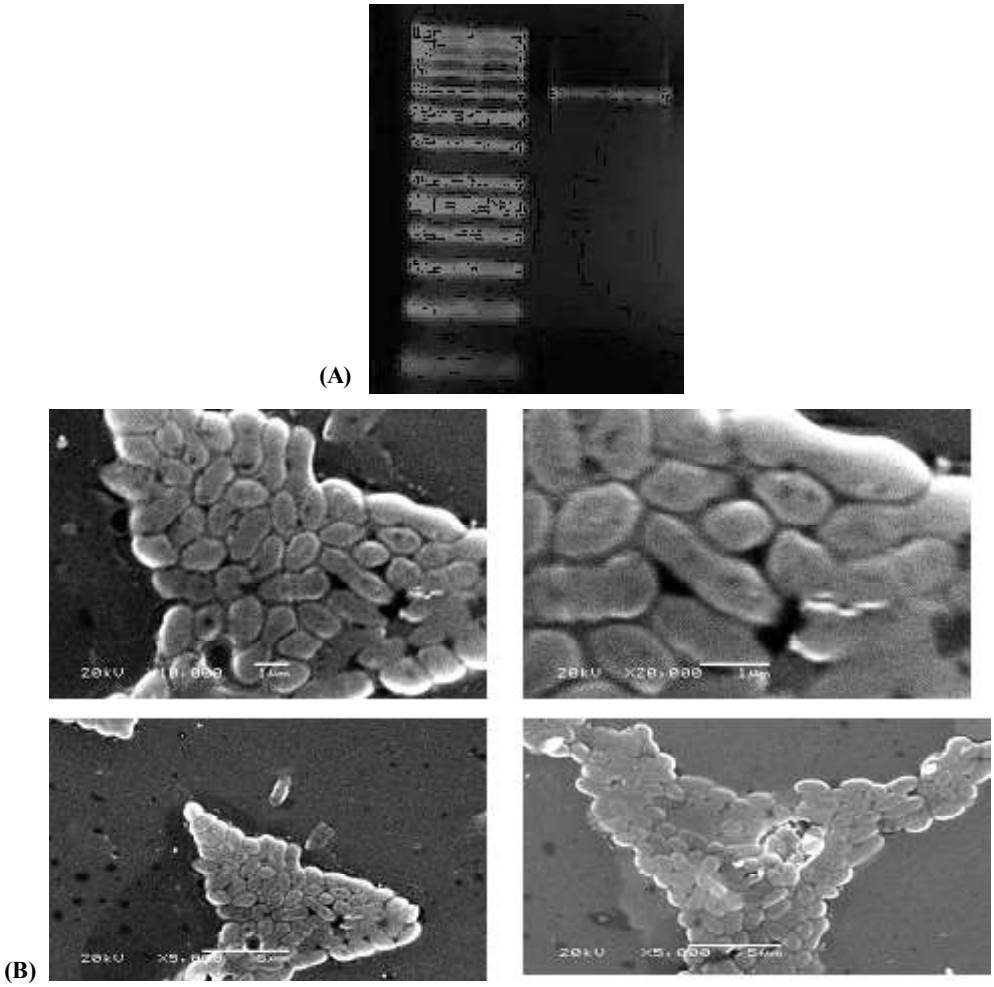

Fig. 1. (a) 16S rDNA amplification products of selected *E. faecium* SFD and DNA ladder gene RulerTM. (b) Scanning electron micrographs of cells Magnification at 10,000 × 1µm, 20,000×~ 1µm and 5,000 × 5µm

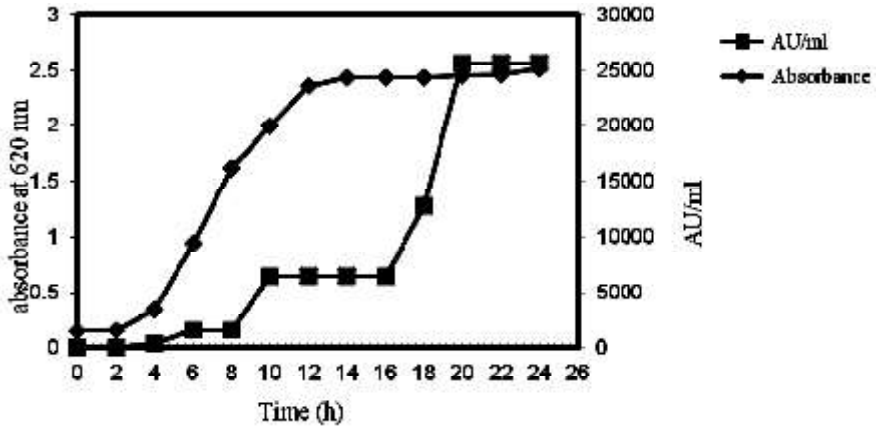

Fig. 2. Change of absorbance at 620 nm (◆—◆) and antibacterial activity of cell-free extract against *Salmonella enterica* ATCC 25566 (■—■) during growth of *E. faecium* SFD in MRS broth at 37°C under uncontrolled pH (initial pH 6.5)

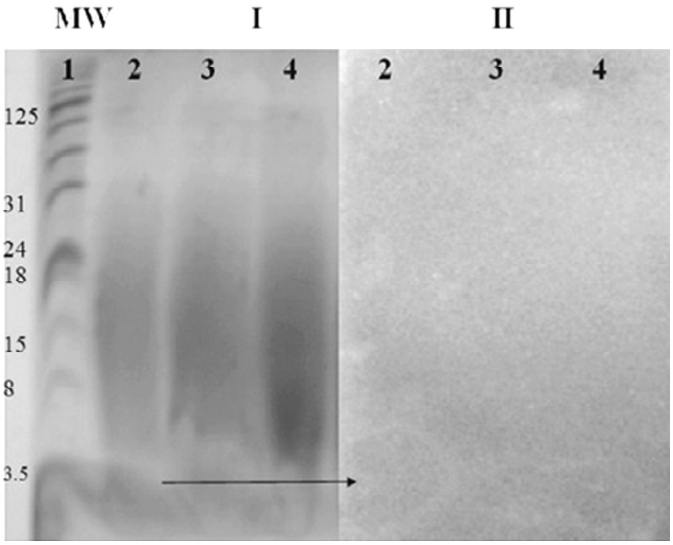

Fig. 3. SDS –PAGE and detection of antibacterial activity of partially purified supernatant of *E. faecium* SFD. I gel stained with coomassie brilliant blue G-250. Lane 1, molecluar weight standards (3.5 to 200 kDa, BioRad), lane 2-4 supernatant after treatment with 30, 40 and 50% saturated ammonium sulphate. II Gel overlaid with cells of *Salmonella enterica* ATCC 25566 inoculated in nutrient soft agar to confirm the bacteriocin band. The inhibition zones after incubation overnight at 37ºC correspond to the coomassie brilliant blue G-250 stained band (indicated by an arrow)

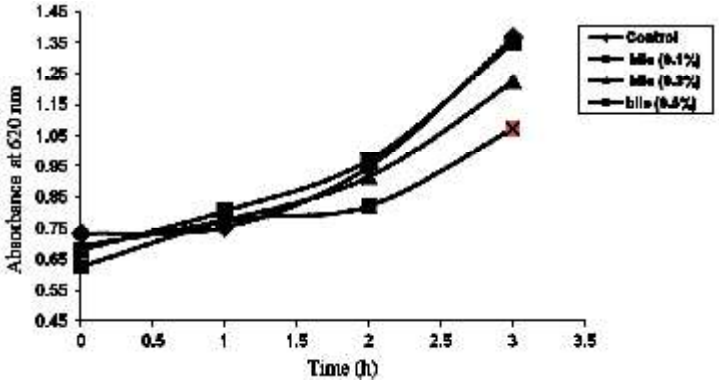

Fig. 4. Growth of *E. faecium* SFD isolate in MRS with and without various bile salt concentrations at 37°C for 3 h expressed in absorbance at 620 nm

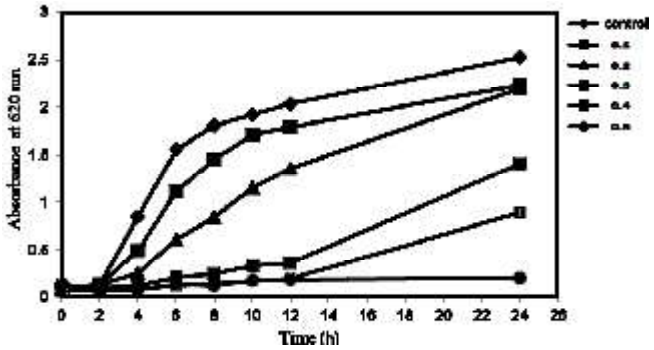

Fig. 5. Tolerance of isolated *E. faecium* SFD grown in MRS broth to various phenol concentrations (0.1 to 0.5%, v/v)

used as indicator strain. However, bacteriocin activity remained unaffected in the presence of detergents; 1% (v/v) Triton X-114, SDS, Tween 20, urea (1%, w/v) and EDTA (0.1 and 0.3 mM) and completely abolished in the presence of 1% DTT and 0.5 mM EDTA when S. *enterica* ATCC 25566 used as indicator strain (Table 4). Similar results were recorded for bacteriocin ST11BR produced by *Lactobacillus paracasei* subsp. *Paracasei* ST11BR[41].

The effect of detergents on different bacteriocins gives information about the structure of the active molecules. Anionic detergents often unfold proteins by complexing to the interior hydrophobic core of their native structure, which may affect their three dimensional conformation. The observed reduction or full loss of bacteriocin activity, following treatment with EDTA (0.5 mM), DTT, Triton X-100, Tween 80 and β-Mercaptoethanol may be due either to partial denaturation or to disruption of its association with other molecules, having a stabilizing effect on its activity[38]. The negative effect of β-Mercaptoethanol on the bacteriocin indicates the presence of at least one disulfide bond in its structure. The characterized bacteriocins of *E. faecium* SFD are mostly class II bacteriocins that have at least one disulfide bond which is necessary for bactericidal activity, e.g., enterocin.

Molecular weight determination

Bacteriocin was partially purified from culture supernatant by ammonium sulphate precipitation and dialysis. A significant increase in the yield of the bacteriocin activity was observed with increasing of salt concentration. The maximum antagonistic activity was found in the resolved precipitate with 70% saturation of ammonium sulfate (819200 AU/ml). Recovered active proteins in the form of pellets were dissolved in phosphate buffer (0.1M, pH- 6.5) and then desalted by dialysis using a 3.5 kDa cut-off membrane against phosphate buffer at 4ºC during 24 h. After dialysis, the bacteriocin activity was found and had not passed the membrane. This indicated that bacteriocin had molecular weight equal or bigger than 3.5 kDa which were confirmed using SDS-PAGE electrophoresis followed by activity analysis (Fig. 3). Other bacteriocins from *E. faecium* strains with close molecular masses have been reported[19] as well as others with higher masses such as enterocin 012[42].

Evaluation of Potential Probiotic Properties

Resistance to gastric acidity

Before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach[43]. The effect of the simulated gastric juice at pH of 2.0 and 2.5 on the viability of the *E. faecium* SFD strains is presented in Table 5. *E. faecium* SFD had a good viability in the simulated gastric juice experiments (which were all replicated twice). After 1 and 2 h at pH 2.5, the number of viable cells had only slightly decreased to 97.34 and 96.30% of the initial viable count, at the respective pH, and the population size was $1.6 \times 10^7 \pm 0.01$ and $1.33 \times 10^7 \pm 0.09$ CFU ml^{-1}, respectively. After 3 h, a 0.28 log cycle reduction in viability was found at pH 2.5 with final populations of $1.33 \times 10^7 \pm 0.03$ CFU ml^{-1}. These results suggest that these

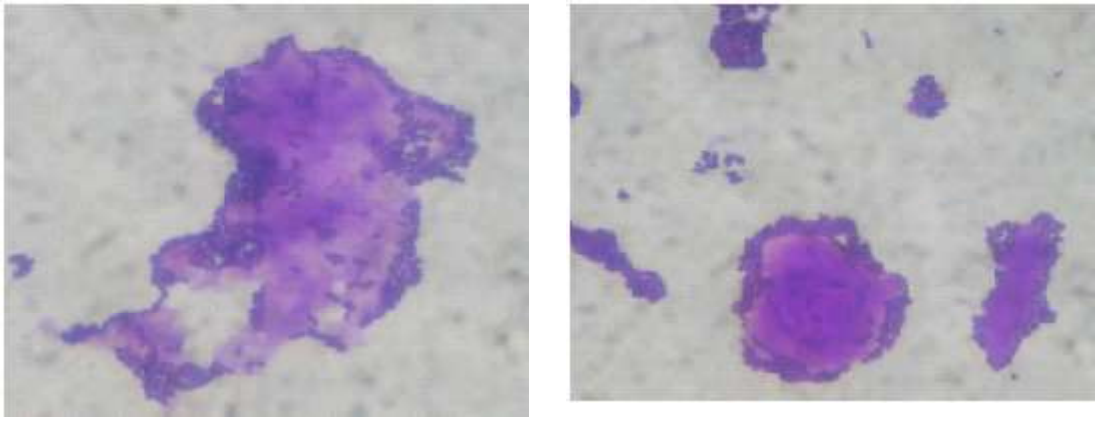

Fig. 6. Microscopy demonstrating the adherence of *E. faecium* SFD to epithelial cells from the small intestines of rabbit (100 magnifications)

human isolates could successfully transit the human stomach and may be capable of reaching the intestinal environment and functioning effectively there. Although the pH in the stomach can be as low as pH 1.0, a high survival rate at pH 2.5 to 3.0 for at least 3 h, is often considered satisfactory, especially as probiotic strains can be buffered by food or other carrier molecules and in fact are not directly exposed to such a low pH in the stomach[44].

Bile salts tolerance

When evaluating the potential of using lactic acid bacteria as effective probiotics it is generally considered necessary to evaluate their ability to resist the effects of bile acids[45]. Bile acids are synthesized in the liver from cholesterol and are secreted from the gall bladder into the duodenum in the conjugated form (500–700 ml/d)[46].

The ability of isolated *E. faecium* SFD to survive for 3 h were examined in the presence of bile salts in concentration ranged from 0.1 to 0.5% (Table 5). The growth of the isolate in MRS broth without bile salts was used as a positive control. *E. faecium* SFD showed full tolerance to all tested concentrations of bile. Cells not only were able to resist different bile concentrations but also slight increase was observed in their growth after 3 h of incubation. After 3 h at bile concentrations of 0.1 and 0.3, the number of viable cells had only slightly increased from the initial viable of $1.15 \times 10^8 \pm 0.55$ CFU ml^{-1}, to $1.9 \times 10^8 \pm 0.62$ and $1.8 \times 10^8 \pm 0.05$ CFU ml^{-1}, respectively. After 3 h, a 0.05 log cycle increase in viability was found at 0.5% bile acids with final populations of $1.33 \times 10^8 \pm 0.062$ CFU ml^{-1}. In addition, growth expressed in absorbance at 620 nm was increased from 0.6 to 1.35; 0.69 to 1.22 and 0.62 to 1.074 after exposure for 3 h for bile concentration of 0.1, 0.3 and 0.5%, respectively (Fig. 4). The average intestinal bile concentration is around 0.3%, and may range up to an extreme of 2.0% during the first hour of digestion[47]. Bile resistance of some strains is related to specific enzyme activity bile salt hydrolase (BSH) which helps to hydrolyze conjugated bile, thus reduce its toxic effect[48]. Hydrolyzation of bile salt by enzyme hydrolases (BSHs) had been explained by Tanaka *et al.*,[49] which can be found in *Enterococcus*[50].

Phenol resistance

For a strain to be a probiotic, it has to survive action of toxic metabolites, primarily phenols, produced during the digestion process, antibiotics and phage, anaerobic growth conditions and storage conditions of the food carrier[51]. Some aromatic amino acids derived from dietary or endogenously produced proteins can be deaminated in the gut by bacteria leading to the formation of phenols[52].

The ability of *E. faecium* SFD to grow in the presence of different phenol concentration ranged from 0.1 to 0.5% was tested. Our results indicate that the selected *E. faecium* SFD showed good tolerance to a phenol concentration of 0.1 to 0.3, while was noticeably inhibited in higher phenol concentration of 0.4 and 0.5% (Fig. 5). Similar findings have been also described previously[53]. High resistance of some *enterococci* strains has been shown[54], while a bacteriostatic effect has been observed for several other strains as well[52].

Hydrophobicity

The interaction of bacterial cells with surrounding environment depends on its surface characteristics such as hydrophobicity, hydrophilicity balance and net charge[55]. The ability to adhere can give information about the possibility of probiotics to colonize and may modulate the host immune system. Several mechanisms were reported about the adhesion of microorganisms to intestinal epithelial cells[56]. Cell hydrophobicity is one of factors that may contribute to adhesion of bacterial cells to host tissues[57]. This property could indicate an advantage and importance for bacterial maintenance in the human gastrointestinal tract[58]. In this study, the *in vitro* determination of microbial adhesion to xylene droplets was carried out. This method was reported to be qualitatively valid to estimate the ability of a strain to adhere to epithelial cells[59]. A hydrophobicity of 37% was determined for isolated *E. faecium* SFD. Hydrophobicity varies among species genetically closely related and even among strains of the same species[60]. Many previous studies on the physicochemistry of microbial cell surfaces have shown that the presence of (glyco-) proteinaceous material at the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides[61] .

Autoaggregation, coaggregation and adhesiveness

Aggregation between microorganisms of the same strain (auto-aggregation), or between different species and strains (co-aggregation), as well as their ability to displace pathogens is an important property of probiotic microorganisms and may have greater advantage over non-co-aggregating organisms which are easily removed from GIT environment.

The autoaggregation rate of *E. faecium* SFD were measured after a period of 4 h. Results showed that the strain exhibited a weak autoaggregating phenotype either under aerobic (14.3%) or anaerobic condition (24.1%). The observed weak autoaggregation could be related to cell surface component, because it may be lost after washing and suspending of the cells in PBS. On contrary to these results, higher autoaggregation was reported for *E. faecium* ST4V (45%), *E. faecium* ST5HA (60%) and *E. faecium* T8 (40%)[62]. Coaggregation of *E. faecium* SFD with another potential probiotic strains (*L. plantarum*) and some other pathogenic bacteria were also examined (Table 6). Results are expressed as the percentage reduction after 1 h in the absorbance of mixed suspension compared with that of the individual suspension. *E. faecium* SFD demonstrated marked coaggregation with *Salmonella enterica* ATCC 25566 (18.5%), *Lactobacillus plantarum* (11.4%) and *Listeria innocua* ATCC 33090 (13.6%) (Table 6). The interaction of probiotic organisms with the natural gut flora is a key to the potential success of the organism in terms of colonization and long-term persistence. Co-aggregation of probiotic bacterial strains has been suggested to enable them to form a physical-chemical barrier that prevents colonization by pathogenic bacteria[63].

Adherent candidate probiotic strains are considered to easily colonize the intestine which is thought to be important for their positive effects[64]. The adhesion of commercial probiotic strains showed a great variability depending on the strain, specie and genera. The adhesiveness of *E. faecium* SFD to intestinal epithelium isolated from rabbit was also investigated. Microscopic examinations showed that this species moderately adhered to epithelial cells (Fig. 5) with adhesion ability of 17.1 (± 3.3) cell adhered to a single epithelial cell. While the tested *E. coli* showed 23.8 ± 3.9 cell adhered to a single epithelial cell.

Cell surface hydrophobicity is a nonspecific interaction between microbial cells and host cells. The initial interaction may be weak, is often reversible and precedes subsequent adhesion processes mediated by more specific mechanisms that involve cell surface proteins and lipoteichoic acid[65]. Bacterial cells with high hydrophobic properties usually form strong interactions with mucosal cells.

Attachment of bacteria to mucosal epithelial cells is considered the initial event in the development of bacterial infections of the gastrointestinal tract. Blocking attachment of pathogenic microorganisms to the intestinal epithelium represents a potential strategy for disease prevention[66]. The interaction between enterococci and different epithelial cells has been analysed previously[67]. Living *E. faecium* 18C23 efficiently inhibited the adhesion of *E. coli* K88ac and K88MB to the piglet intestinal mucus[68]. This investigation has shown that *E. faecium* SFD has the ability to establish in the human gastrointestinal tract, which is an important determinant in the choice of probiotic strains.

Cholesterol assimilation

Elevated serum cholesterol in humans is generally a risk factor correlated with development of coronary heart disease. Modification of diets such as supplementation of diet with fermented dairy products or LAB-containing dairy products is a way that may be helpful in reducing serum cholesterol[69].The tested isolate of *E. faecium* SFD decreased cholesterol concentration from 110 ± 2.2 mg/dl to 71.4 mg/dl, in the 0.3% bile salt MRS broth with removal percentage of 35 (Table 6). LAB may alter serum cholesterol by two proposed mechanisms; directly binding of dietary cholesterol in the small intestine before cholesterol can be absorbed into the body[70] and deconjugation of bile acids to produce free bile acids[71]. However, the most frequently suggested mechanisms of lactic acid bacteria activity on cholesterol level are adhesion of the cholesterol to the cell surface and incorporation of the cholesterol into the cellular membrane[72,73]. The present study suggests that consumption of *E. faecium* SFD will be useful in reducing human serum cholesterol level. However, additional *in vitro* and *in vivo* studies are needed.

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