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Partial Purificatin of Bulgaricin Antibacterial from *Lactobacilli* Isolates in Iraqi Kurdish Dairy Product

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Abstract. Objectives: To obtain new strain of beneficial Lactobacilli with higher antibacterial activity as a bacterial therapeutic bulgaricin. To optimize growth of bulgaricin production. **Methods:** Seventy five isolates of Lactobacillus were obtained from traditional Kurdish dairy products including: 25 isolates from raw milk, 20 from cheese, and 30 from yoghourt. Controlling of bacteria growth and bulgaricin production were achieved using specific temperature and acidic medium. 60% of ammonium sulphate saturation was used for bulgaricin partial purification, and gel filtration using sephsdex G-100 column was applied for further purification of bulgaricin fraction. Electrophoretic analysis was applied using SDS-Page for the determination of the molecular size of bulgaricin. **Results:** Sixty nine of the 75 isolates were possessed strong antibacterial activity, and six of them were identified as bulgaricin. Among these isolates, Y_{20} was selected as specific according to its antibacterial action on four indicator bacteria and due to its higher productivity of bulgaricin. The production was achieved using 30°C and pH value of 4. The molecular size obtained was 22kD, and the isoelectric point was 6.2. **Aims:** To assess the use and addition of bacteriocinogenic strains as probiotic to food stuff like yoghourt product as an industrial starter culture. **Conclusion:** The results of the study revealed the probability of using bulgaricin growth in food and food producers since they can kill or inhibit pathogenic bacteria.

Keywords: Bacteriocin, lactobacillus, Partial purification.

1. Introduction

Bacteriocins (Bcn) (ribosomal peptidic antibiotics) comprise a large and diverse group of heterologus subgroup of small ribosomally synthesized bioactive antimicrobial proteins or peptides of bacterial origin that were originally defined as the proteinaceous compounds, some of these which undergo post-translational modifications whereas others are not modified. These compounds have a bactericidal or bacteriostatic mode of action and inhibitory almost exclusively toward other sensitive strains or species of Gram-positive bacteria and particularly toward taxonomically closely related species to producer bacterium, however, few exceptions to the rule, i.e. activity against Gram-negative bacteria, have been reported [1-3]. Also Bcn_s are plasmid-mediated, reactive with specific binding sites on sensitive bacteria, produced by lethal biosynthesis and characterized by a narrow range of sensitive organisms [4]. Studies have demonstrated that few antagonistic substances, especially those produced by Gram-positive bacteria, fit adequately and closely to the classical definition of bacteriocins produced by Gram-negative bacteria (classical colicin model) [5-7] suggested that the definition of bacteriocins should be based on only two basic requisted, i.e. their protein nature and the absence of lethality to the producer cells. These authors confirmed that few antimicrobial proteins fit the classical definition proposed by Tagg *etal.* [5]. According to Klaenhammer [8] and Jack *etal.* [9], Bcn_s are extracellularly released low molecular weight, heat stable, ribosomally synthesized, and

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cationic proteinaceous compound, produced by Gram-positive organisms, with antibiotic-like functionally against closely related species by adsorption to receptors on the target cells. Bacteriocins do not respond to well defined criteria. Rather, their biochemical properties, molecular weight, spectrum of activity, mode of action and genetic support are very heterogenous [10]. Bradley [11], considered Bcn_s defective bacteriophages, which lack the ability to multiply intracellularly but are able to lyse a sensitive cell, to be physiologically identical to Bcn_s. In other cases, enzymes with either hemolysin, phospholipase, or bacteriolytic activities have also been categorized as bacteriocins [9]. Several bacteriocins have been biochemically and genetically characterized [12]. Members of the genus *Lactobacillus* are known to secrete bacteriocins [13]. It is well known that *Lactobacillus* spp. produce certain antimicrobial substances, including antibacterial peptides (bacteriocins). Several bacteriocins from *Lactobacillus* spp. have been characterized with respect to their protein sequence, molecular mass, biochemical properties and antimicrobial activity spectrum. Bacteriocins that have all D-amino acids have antibacterial activity but exhibit more resistance to proteolytic enzymes and are less cytotoxic compared with bacteriocins that have all L-amino acids [14]. The interest in the application of microorganisms and their metabolites in the prevention of the food spoilage and the extension of the shelf life of foods have been increased during the past decade [15, 16]. Most of the bacteriocin producing LAB are isolated from fermented food [3]. Lactic acid bacteria often produce more than one bacteriocin. Detailed structure analyses have confirmed that in some cases of the bacteriocins are entirely different peptides and in others are breakdown products or oxidized forms of one bacteriocin. Bacteriocins produced by LAB were intensively investigated. In addition to typical LAB bacteriocins with a narrow antibacterial spectrum, but LAB also that produce bacteriocins having a wider or broad antibacterial spectrum have been described. Thus, some LAB bacteriocins can inhibit the growth of Gram-positive pathogenic and spoilage bacteria as well yeasts. Additionally, it has been reported that bacteriocins also inhibit the growth of some Gram-negative species [15, 16]. The aims of this study was to Produce a bacterial therapeutic proteins (bacteriocins) with new characteristics to control antibiotic-resistant bacteria and bacteriotherapy.

2. Materials and Methods

2.1. Sample Collection

Ninety samples represents traditional Kurdish dairy products (milk, cheese, and yogurt) were collected from April 2008 till March 2009, from local retail markets of different areas in Sulaimani city in sterilized, isothermic and insulated containers. Samples were immediately cooled for further microbiological analysis. Thirty Lactobacillus isolates were selected due to their ability of producing antibacterial activity, and six of them were producing bulgaricin. One milliliter of *Lactobacillus* broth culture was inoculated into each carbohydrate fermentation medium. To ensure anaerobic conditions, each tube was supplemented with two drops of sterile liquid paraffin after inoculation. Samples were incubated anaerobically and carbohydrates utilization was assessed at the 24 and 48 h at 37 ºC. In the case of ability of sugar fermentation, the color was changed from red to yellow reflecting the test as positive [17].

2.2. Screening and Detection of Bacteriocin-Producing *Lactobacillus* Isolates

Preliminary production of bacteriocin by the isolated *Lactobacillus* was screened by the same method described previously [18]. To find out bacteriocinogenic isolates of *Lactobacillus*, cell-free supernatant was adjusted to pH 7 to exclude the antimicrobial effect of organic acids and rule out any inhibition due to pH reduction caused by organic acid production, Anaerobic was used to rule out any inhibition due to hydrogen peroxide production [19]. Observing of inhibitory activity of neutralized cell-free supernatants indicates the action of bacteriocins and bacteriocin-like metabolites.

2.3. Isolation of Lactobacilli

Five grams of yoghurt was taken aseptically and transferred to sterile plastic bags and homogenized in 45 ml. of sterile 0.1% (w/v) peptone water or buffered peptone water as diluent and 10-fold serially diluted. The isolation was performed by the routine microbiological procedure and inoculation on a solid medium using MRS agar plates as a selective media for Lactobacilli isolation. A volume of 0.1 ml of appropriate dilutions of the sample was pour-plated and spread in duplicate onto MRS medium, then inoculated plates

were incubated anaerobically at 37 ºC for 24-48 h in an anaerobic jar in the presence of a Gas Generating Kit and copper coated steel wool* [20]. Plates were re-incubated for further overnight before discarding them as negative. The purified isolates were stored and maintained frozen at -40 ºC in MRS broth containing 25% glycerol (v/v) for further analyses and subcultured every six months [21]. The suspected *Lactobacillus* isolates were identified following the methods in Bergey's Manual of Systematic Bacteriology, Vol. 2 [22], The Genera of Lactic Acid Bacteria, Vol. 2 [23] and Critical Reviews in Microbiology [24]. The isolate were initially subjected to Gram reaction, all being Gram-positive were considered as *Lactobacillus* and tested for further studies. Gram stain and morphology were examined after 24 h incubation on MRS agar [25].

2.4. Partial Purification of Bcn Isolate

A protocol of Todorov and Dicks, [1] and Karthikeyan and Santosh, [26], have been adopted in the partial purification of Bcn. Isolate. The crude bacteriocin sample produced was partially purified by treating the crude three rounds with 60% solid ammonium sulphate. The precipitates formed were separated by centrifugation, dissolved and 2mls of its solution were loaded on to a column (1.5x75 cm) chromatography Gel-filtration using sephadex G-100 and eluted with phosphate buffer pH 7 at flow rate of 0.2 ml/min. Electrophoresis analysis was applied for the purified peak (150µl) using SDS gel plate as matrix. Estimation of the molecular size, standard proteins of different molecular weights including pepsin, ovalbumin, catalase, and bovine serum albumin (BSA) were used (fig-3b). The molecular mass of bulgaricin appeared (fig-3a) to be approximately 22 kDa as compared to the mobility of the standard proteins on a logarithmic plot.

2.5. Preservative Effect of the Bulgaricin against Different Bacterial Species Compared to Some Natural Plant Extracts and Organic Acids

Rosemary essential oil (leaf) and acorn extracts of two species of the oak tree were extracted by Clevenger and Soxhelt (petroleum spirit extraction) equipment, respectively, in the laboratories of Sulaimani University. Antimicrobial activity of the purified bulgaricin from Y_{20} isolate against four bacterial species was determined by agar well diffusion assay. The inhibitory activity was tested on soft Mueller-Hinton agar (0.75% w/v) plate inoculated with an overnight culture of the test microorganisms. Plates were kept at cool temperature (4 ºC) for 2-4 h and then incubated at 37 ºC for 24 hours. Antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells. Test bacteria were two isolates of each *Bacilluscereus*, *Escherichiacoli*, *Salmonella*, and *Staphylococcusaureus* and each isolated from different sources including clinical, food, soil samples [27, 28].

3. Results

Figure -1: shows the protein fraction of Bcn absorbency from the dialyzed crude (Y20).

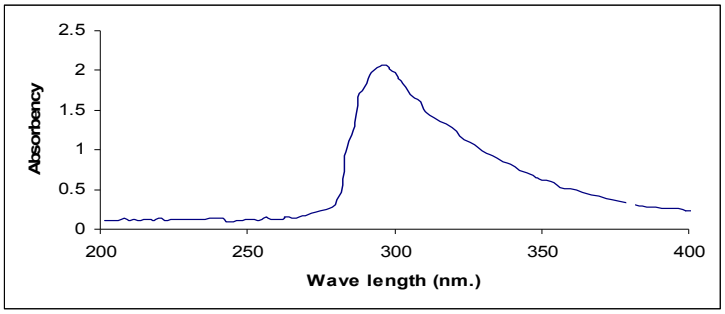

Fig. 1: Absorbency readings of the CFS of Y_{20} isolate at wavelength between 200 to 400 nanometer.

Figure (2), shows the elution pattern of the dialysed Bcn using sephadex G-100 column gel filtration. Active peak of Bcn was purified between fractions 16 and 30.

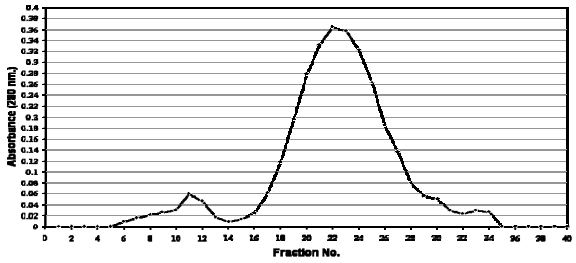

Fig. 2: Purification of the bulgaricin by Sephadex G-100 column.

The molecular weight of Bcn peak was determined using electrophoresis analysis (fig-3a) and compared with pepsin, ovalbumin, catalae, and BSA standered proteins (fig-3b).

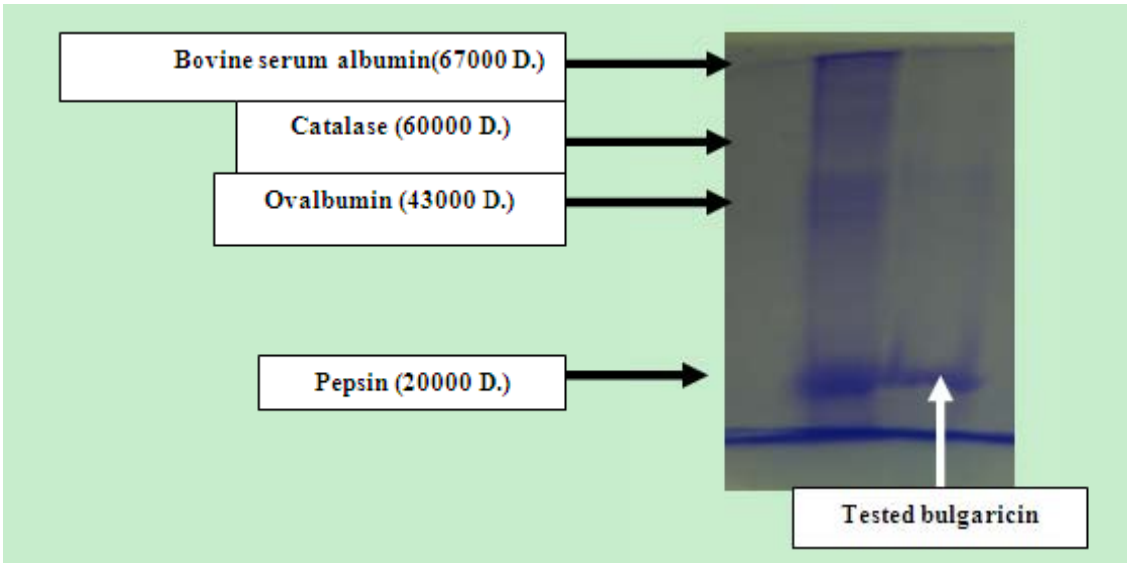

Fig. 3a: Electrophoretogram of SDS-PAGE of the bulgaricin.

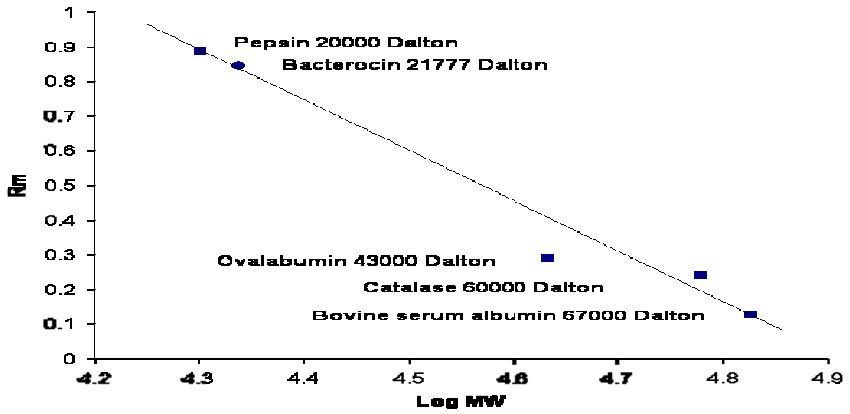

Fig. 3b: Molecular size of the bulgaricin

Applying isoelectric focusing technique, the isoelectric point of Bcn (Y20) appeared to be around 6.2 (fig-4a).

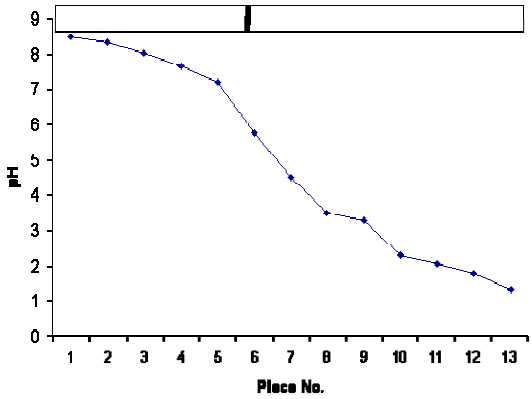

Fig. 4a: Determination of the isoelectric point (pI) for the bulgaricin.

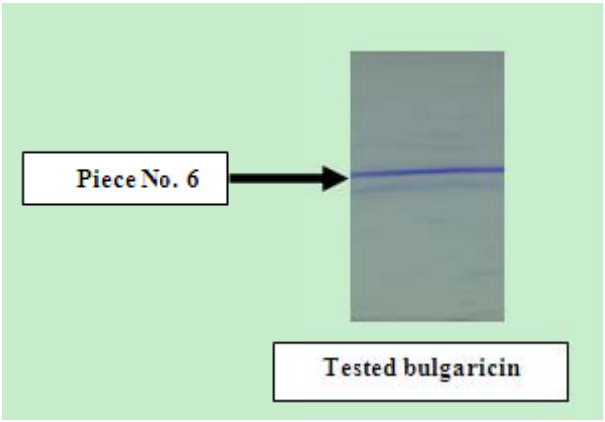

Fig. 4b : Determination of the isoelectric point (pI) of the bulgaricin.

Table 1: Effect of used different concentrations of ammonium sulphate (fractionation) on antibacterial activity of bacteriocin from *Lactobacillus* isolate (Y_{20}).

Saturation with $(NH_4)_2SO_4$ (%)	**Inhibition zone diameter/mm***			
	B. cereus	***E. coli***	***P. aeruginosa***	***S. aureus***
50	10	10	9.5	11
60	12	14	11	13
70	11	12	10	12

* 5 mm well diameter is included.

Table 2: Samples of raw milk, white soft cheese and yoghurt positive for *Lactobacill*

Food source		**Number of samples**
	Total	***Lactobacillus* positive**
Raw milk	30	25 (83.33%)
White soft cheese	30	20 (66.67%)
Yoghurt	30	30 (100%)
All	90	75 (83.33%)

Table 3: Comparative study for the effectiveness of natural plant extract products, organic acids and bulgaricinagainst different bacterial isolates.

Substance	Diameter of inhibition zone (mm)*							
	***B. cereus*¤**		***E. coli*†**		***Salmonella*Ψ**		***S.aureus*φ**	
	1	**2**	**1**	**2**	**1**	**2**	**1**	**2**
Bulgaricin	13	12	13	14	0	0	15	15
Rosemary essential oil (*Rosmarinusofficinalis*)	30	25	0	0	15	13	15	14
A corn(*Quercusinfectoria*)	0	0	0	6	12	0	14	12
A corn (*Quercusbrantii*)	8	0	0	0	0	0	0	0
Acetic acid (10%)	7	6	7.5	7	8	8	9	8
Lactic acid (10%)	5.5	6	6	6	5.5	6	6.5	6
Fennel essential oil (*Foeniculumvulgare*)	0	0	0	0	0	0	11	0
Menthol (*Mentha* sp.)	0	0	45	0	9	10	20	0

***** 5 mm well diameter is included.

¤***B. cereus* isolate** (1-dried food), (2-soil). ***E.coli*** (1-soft cheese), (2-clinical samples).

Ψ***Salmonella* isolate** (1-minced meat), (20clinical samples). ***S. aureus*** (1-raw cow milk). (2-sheep with mastitis).

4. Discussion

4.1. Confirmation of antimicrobial substance produced by Y_{20} isolate as bacteriocin

Cell-free supernatant obtained from designated Y_{20} isolate was subjected to the absorbency measurement, reading were carried out between two wave lengths (200-400nm) with intervals of 0.5 (figure-1). Generally, proteinaceous materials gives absorbency readings at 280 nm (A_{280}) and as shown in the figure if a straight line dropped from the top of the peak downfall on the value 290 nm indicates that the solution contains proteins (crude) which has been mixed with other materials.

4.2. Partial Purification of Bulgaricin Produced by *L. Bulgaricus* (Y_{20} Isolate)

An increase in antimicrobial activity after partial purification of crude bacteriocin by ammonium sulphate (60%) precipitation observed with four indicator strains (Table-1). Highest and maximum bacteriocin inhibitory activity was observed. precipitated with 60% . Such technique was also applied by others to purify partially various bacteriocins from different lactic acid bacteria by using the salt ammonium sulphate. Kim *et al.* [29], partially purified a bacteriocin produced by *Lactobacillus bulgaricus* using 40% ammonium sulphate as final concentration. Also the bacteriocin isolated from malted barely was precipitated from cell free supernatant using 40% ammonium sulphate [30]. Karthikeyan and Santosh [26], measured the maximum inhibitory activity of a bacteriocin from *L. plantarum* at 50% saturation. According to Joshi *et al.* [31], the highest bacteriocin activity from natural lactic acid fermentation of vegetables was obtained with the use of 20-30% ammonium sulphate. Purification of bacteriocins might be affected by some factors. Ammonium sulphate precipitation of culture supernatants containing Tween 80 resulted in 3 distinct phases after centrifugation, a surface pellicle, a bottom pellet, and the supernatant [32-34]. Majority of activity of lactacin F produced by *L. acidophilus* 11088 was recovered in the surface pellicle [32], whereas, lactacin F and lactocin S produced by *L. sake* L45 [33] were lost in the floating pellet. Therefore these authors suggested to collect pellicle instead of the pellet for further purification. During the purification, bacteriocins become unstable due to loss of cofactors, or modifications on the catalytic site [35].

4.3. Recovery of Bulgaricin in Dialysates

Bulgaricin permeated dialysis tubing with a molecular weight cut-off of 3.5 kDa (3.5 kg mol^{-1}) against 1000 ml phosphate buffer pH 7 for an overnight. Hastings *et al.* [36], reported a loss of activity due to dialysis even when a membrane with a 2000 Dalton cut-off was used. The authors also reported the importance of pH during purification. Techniques with large change in pH generally cause great losses in activity of bacteriocins and low pH (pH 2-4) during the procedure resulted in good yields.

4.4. Molecular size of the Bulgaricin by SDS-PAGE

For the estimation of the molecular size of bulgaricin, standard proteins of different molecular weights including pepsin, ovalbumin, catalase, and bovine serum albumin (BSA) were used (Figure-3b). The apparent molecular weight of the bulgaricin was shown in figure (3a), the bulgaricin was migrated as a clear single band with a mobility slightly higher than pepsin and when the mobility of the bulgaricin was compared to the mobility of the standard proteins with different molecular weights on the logarithmic plot, the molecular mass was appeared to be approximately 22 kDa. While the bacteriocins characterized from Gram-positive species are predominantly small (<10 kDa) peptides, several large antimicrobial proteins have been described at both biochemical and genetic level. These bacteriocins typically manifest as heat-labile proteins, but one apparent exception is propionicin SM1, a heat-stable inhibitory agent produced by *Propionibacterium jensenii* [37].

4.5. Isoelectric Focusing (IEF) for the Bulgaricin

The isoelectric point of the bulgaricin was appeared to be around 6.2 (figure -4a & 4b). The high isoelectric point allows bacteriocins to interact at physiological pH values with the anionic surface of bacterial membranes. This interaction can suffice, in the case of broad-spectrum bacteriocins, or facilitate, in the case of receptor-requiring compounds, insertion of the hydrophobic moiety into the bacterial membrane. Later, the cooperation between a numbers of bacteriocin molecules will build up the transmembrane pore responsible for gradient dissipation and cellular death [38].

4.6. Preservative Effect of the Bulgaricin against Different Bacterial Species Compared to Some Natural Plant Extracts and Organic Acids

The *in vitro* antibacterial activities of the bacteriocin, natural plant extracts and organic acids are shown in Table (3). Determination of the inhibition zones by means of the agar well diffusion method showed that the different tested agents exhibited different antibacterial effects against the four tested bacteria. According to the current study, the bacteriocin, rosemary essential oil, and menthol were more effective against the four bacterial species especially against *E.coli* and *S.aureus*. Similar results were obtained by Erdoğrul and Erbilir [39] where the antimicrobial activity of *L.bulgaricus* bacteriocins isolated from dairy products against *E.coli*, *Salmonella typhimurium*, and *S.aureus* were 8, 7, and 8 mm, respectively. Concerning antimicrobial activity of the plant extracts, Abu-Shanab *etal.* [27], reported the inhibitory properties of *Rosmarinu sofficinalis* extract on different bacteria including entero hemorrhagic *E.coli* O157 (EHEC) and Methicillin-resistant *Staphylococcus aureus* (MRSA), while no effect was detected for entero hemorrhagic *E.coli* (EHEC). An inhibition zone of 12 mm was observed from hot water extracts against MRSA. Abed [40], found no effect for the fennel volatile oil extracted either by water steam distillation or simple solvent extraction against *S.aureus* ATCC 25923, *E.coli* ATCC 25922, and *P.aeruginosa*. While the bacteriocins characterized from Gram-positive species are predominantly small (<10 kDa) peptides, several large antimicrobial proteins have been described at both biochemical and genetic level. These bacteriocins typically manifest as heat-labile proteins, but one apparent exception is propionicin SM1, a heat-stable inhibitory agent produced by *Propionibacteriumjensenii* [37].

5. Conclusion

Bacteriocin production could be considered as an advantage for food and food producers since, in sufficient amounts, these peptides can kill or inhibit pathogenic bacteria that compete for the same ecological niche or nutrient pool. Although, many of the ready to eat and novel food types represent new food systems with respect to health risks and spoilage. Vegetarian foods and products have frequently been found to be contaminated with various food spoilage and pathogenic organisms. A recent trend is to use and add

bacteriocinogenic strains as probiotics to fermented milks especially to yoghurt (probiotic milks; yoghurt probiotic milks) because *L.delbrueckii* subsp. *bulgaricus* is one of the two bacteria necessary for the yoghurt production as industrial starter culture.

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