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RESEARCH ARTICLE

Combined Effect of Nanoparticles and *Leuconostoc mesentroides ssp. cremoris* Bacteriocin against *Listeria monocytogenes* Isolated from Locally Soft Cheese

Zahraa A. Khadam[1], Zinab Owaid Shatti[1], Nibras Nazar Mahmood[1*], Mohammed F. Al-Marjani[1], Jehan Abdul Sattar Salman[1], Raghad A. Aziz[2], Khalid Waleed Qassim[3]

1. *College of Science-Mustansiriyah University. Baghdad-Iraq.*
2. *College of Basic Education-Mustansiriyah University. Baghdad-Iraq.*
3. *College of medicine Al-Nahrain University, Baghdad-Iraq.*

***Corresponding Author: Nibras Nazar Mahmood**

Abstract

Listeria monocytogenes has been as important opportunistic bacteria for human since 1929 and as food borne pathogenic bacteria since 1981. Out of Twenty samples of locally soft cheese, only two isolates of *Listeria monocytogenes* were isolated and identified using microscopically, cultural and biochemical methods. The isolates were multi drug resistant to ceftriaxone ,trimethoprime amoxicillin ,aztreoname, ciprofloxacin and penicillin. While, these isolates were sensitive to Ampicilin, imipenem, and azithromycin. The antilisterial activity of 400 µg/ml of both silver and zinc Nanoparticles and bacteriocin of *L. mesentroides ssp. cremoris* in soft cheese for 24 h. was evaluated. The results showed that bacteriocin, silver nanoparticles and zinc nanoparticles reduced the growth of *L.monocytogenes* in cheese with inhibition percent (49.48, 19 .38, 38.14) % respectively .The combination between bacteriocin with nanoparticles (1:1) showed increased in the reduction of *L.monocytogenes* growth with (58.76 and 67.01) % for bacteriocin with silver nanoparticles and bacteriocin with zinc nanoparticles respectively. These results indicate that the combination of bacteriocin with nanoparticles has a protective effect against *Listeria monocytogenes* in locally soft cheese

Keywords: *Leuconostoc mesentroides*; Cheese; *Listeria monocytogenes*; Nanoparticles.

Introduction

Listeria monocytogenes is a Gram+ve pathogenic bacteria that can cause listeriosis acute invasive disease, mainly in immunocompromised patients, elderly persons, and pregnant women, characterized by sepsis, meningitis and miscarriage [1]. Listeriosis has now been identified as an emerging food borne zoo noses [2] *Listeria monocytogenes* is an important food borne pathogenic bacteria that can be isolated from food processing and products [3].

Behavior that make *Listeria monocytogenes* difficult to be controlled during food processing it's their ability to increase over an extensive range of temperatures mainly refrigerator temperatures, resist to different environmental stresses, form biofilms, and ability to contaminate food products [4].Nanotechnology has emerged up as a good technology for particles synthesis in the nanometer size during the past few decades, which reveals antibacterial activity due to their high surface area to-volume ratio and unique physical and chemical properties. The bactericidal activity of different metallic nanoparticles including titanium, copper, silver and zinc, have been well recognized [5].

The main advantage of Ag nanoparticles is that they have an inhibitory activity on bacterial cells, which has led to wide study attempting to understand the various methods involved in these effects [6]. Microorganisms can assemble a resistance against some antimicrobial agents, but not silver ions because silver attacks a wide range of bacterial targets [7].

Lactic acid bacteria isolates showed antagonistic activity against *Listeria monocytogenes* [8]. Extracellular protein inhibitor produced by lactic acid bacteria, including *Leuconostoc*, and has an effective effect against food borne pathogens by inhibiting the structure of plasma membrane proteins and inhibition of cell nucleic acids [9].In this study, the effectiveness of nanoparticles and *L.mesentroides ssp. cremoris* bacteriocin in soft cheese on the survival and growth of *L. monocytogenes* in soft cheese was evaluated.

Methods

Isolation of *Listeria Monocytogenes* from Locally Soft Cheese

Twenty samples of soft cheese was collected from the local market and transferred immediately to the laboratory. The suspension of cheese (0.5 ml) was inoculated in a sterile test tube containing BHI broth (10 ml) which incubated for 24 h. at 37 C^{o} ,then (0.5) ml of the bacterial culture was inoculated on nutrient agar, blood agar, and PALCAM agar ,then incubated for 24 h. at 37 C^{o}. A single pure colony was taken from culture and its examination of the morphological properties, microscopic examination, and biochemical tests were done for the final identification.

Isolation and Identification of *Leuconostoc Mesenteroides*

For *Leuconostoc mesenteroides* isolation, each sample of raw cow milk was inoculated in MRS broth at (1%) and cultivated at 30^{o}C for 24 h under anaerobic condition. A series of dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) were made for each sample with phosphate buffer saline, then spread (0.1) ml of the fourth dilution on solid MRS Vancomycin agar plates [10].

MRS Vancomycin agar plates were incubated at 30^{o}C for 24 h anaerobically. Sub culturing of isolated colonies on MRS agar for further purification, incubated under anaerobic condition at 30^{o}C for 24 h, then the individual colonies were transported to MRS broth and incubated under the same conditions. The purified isolates were identified through microscopically, cultural and biochemical tests [11] and Vitek2 system.

Antibiotic Susceptibility Test of *Listeria Monocytogenes*

Disk diffusion test was performed to test the sensitivity of *L. monocytogenes* isolates to different antibiotics on Mueller-Hinton agar with inoculums compared with McFarland turbidity (0.5) according to CLSI. The susceptibility of the isolates was determined against antibacterial agents included: ceftriaxone, trimethoprime amoxicillin, aztreoname, ciprofloxacin, penicillin, Ampicilin, imipenem, and azithromycin.

Extraction of Bacteriocin from *Leuconostoc mesenteroides* ssp. *cremoris*

The bacterial isolate was inoculated in MRS broth, incubated at 30C for 24h. Bacteriocin was obtained by centrifugation (10,000 rpm for 15 min), sterilized by Millipore filter (0.02 μm), the supernatant was neutralized to pH. 6.2 By adding 1N NaOH to remove the influence of organic acid (12).The crude bacteriocin was concentrated using Dialysis bags and poly ethylene glycol 20000 (13).

Purification of Bacteriocin Produced by *L. mesenteroide* ssp. *cremoris* using Sepacryl S-200 gel

The gel (supplied by Swedish Pharmacia) washed with the solution of the phosphate buffer and perform the degassing step, Then the gel was filled in a column to give a final 1.5 x 60 cm to avoid the formation of air bubbles, after making sure to reach the required dimensions, the column balancing was performed for 24 hours to confirm the required pressure of the separation material.

After calculating the required flow velocity and setting the column mode, then added the concentrated supernatant to the top of the column then the separate parts were then collected at 3 millimeters / part, and then read the separate parts with spectrophotometer at 280 nm. The concentration of the quantitative protein of the separate peaks was estimated.

Silver and Zinc Nanoparticles

Silver and Zinc nanoparticles powder (20nm) with purity (99.99%) used in this study supplied from (hongwu nanometer).

Determination of Minimum Inhibitory Concentration (MIC) for Bacteriocin and Nanoparticles

The MIC of bacteriocin, silver and zinc nanoparticles was determined by the micro

dilution method in the culture broth [14] with modification.

Further dilutions were prepared to concentrations ranging from (600-50) µg/ml. All the wells of the 96-well micro plate were inoculated with 2.5 µl of an overnight culture (1.5×10^8 CFU/ ml) of *Listeria monocytogenes* isolates. Micro plates were covered and incubated for 24 h. at 37 °C. The minimum inhibitory concentration was determined at a concentration which no visible growth could be observed after sub culturing on Nutrient agar.

Combined Effect of Nanoparticles and Bacteriocin against *L. monocytogenes*

Five gm. of cheese sample was inoculated into DW and sterilized for 15 min, it was inoculated after cooling with *Listeria monocytogenes* (10^5cell/ml), then homogenized for 15 min. The antilisterial effect of *L. mesentroides ssp. cremoris* bacteriocin (400 µg/ml) and nanoparticles (400µg/ml for each silver and zinc) was quantified by adding 1 ml from each treatments, separately to homogenized *L. monocytogenes*.

The *L.monocytogenes* homogenizer without bacteriocin or nanoparticles was used as control. All the treatments and control were incubated for 24h. at 37°C. After incubation period, serial tenfold dilutions (up to10^{-6}) was made, 0.1 ml of each dilution was cultured on nutrient agar and incubated for(24-48) h. at 37°C. The colonies were counted and the reduction of *L. monocytogenes* growth was evaluated for all treatments using the following equation described as [15].

$$\mathbf{R\ (\%) = [A-B]/A \times 100}$$

R: the rate of reduction: A: the number of *L. monocytogenes* colonies from control medium and B= the number of *L. monocytogenes* colonies from treatments.

Results and Discussion

Listeria monocytogenes is a food borne pathogenic bacteria that is present in the environment, causative listeriosis, a fatal disease, particularly for immunocompromised [16].Food borne disease poses a significant health problem in the world, with significant rate of mortality and morbidity, in both developing and developed countries [17].Out of twenty soft cheese samples collected from the local market in Baghdad , only two isolates of *L. monocytogenes* were isolated and identified using morphological ,microscopically , and biochemical tests. *L. monocytogenes* grows as grayish-green colonies surrounded by black halos on PALCAM agar [18, 19]. The methods for detecting the food borne bacteria present in food are depend on culturing the bacteria on agar medium followed by biochemical identifications [20].

Antibiotic susceptibility test of *Listeria monocytogenes*

Listeria monocytogenes isolates were tested for their antibiotic susceptibility to different antibiotics groups, using disc-diffusion method, the isolates were MDR to ceftriaxone, trimethoprime amoxicillin, aztreoname, ciprofloxacin and penicillin. While, these isolates were sensitive to Ampicilin, impanel, and azithromycin (Table 1). The results of antimicrobial sensitivity test in current study were in agreement with the finding of Marius *et al* [21]. Who revealed that the drugs of choice for *L. monocytogenes* infections were ampicillin with gentamicin or penicillin.

The results of current study are also in agreement with Temple *et al.* [22] who showed that ampicilin is the drug of choice for the *L. monocytogenes* treatment. The differences in pattern of antimicrobial sensitivity of *L. monocytogenes* might be due to the gaining of the antibiotic resistance genes from other G+ve bacteria by transposable genes and plasmids [23]. Till now, group of ß-lactams is the best in listeriosis treatment because the ability in penetrating the macrophages [24].

Table 1: Antibiotic susceptibility of *Listeria monocytogenes* isolates.

Isolates	CRO	ATM	TMP	P	AMC	IMP	CIP	AP	AZM
***L. monocytogenes*(1)**	R	R	R	R	R	S	R	S	S
***L. monocytogenes*(1)**	R	R	R	R	R	S	S	S	S

Ceftriaxone (CRO) , aztreoname (ATM) , trimethoprime(TMP) ,penicillin (P) ,amoxicillin (AMC), imipenem(IMP), ciprofloxacin(CIP), Ampicilin (Ap), and azithromycin (AZM),R :resistance, S: sensitive

Purification of Bacteriocin using Gel Filtration

Sephacryl S-200 was used to purify bacteriocin [13], the sample obtained after concentration with polyethylene glycol 20000 was added to the gel filtration column with a dimension of 1.5 x 60 cm, to purify the bacteriocin from *L. mesenteroides* ssp. *cremoris*. To verify the accuracy of the purification process of the separated proteins, electrophoresis was carried out with polyacrylamide gel and the presence of the auxiliary factors. The results showed the emergence of a single package of purified bacteriocin from *L. mesenteroides* ssp. *cremoris*. The protein ranged from 0.596 to 1.941 mg / ml (Figure 1).

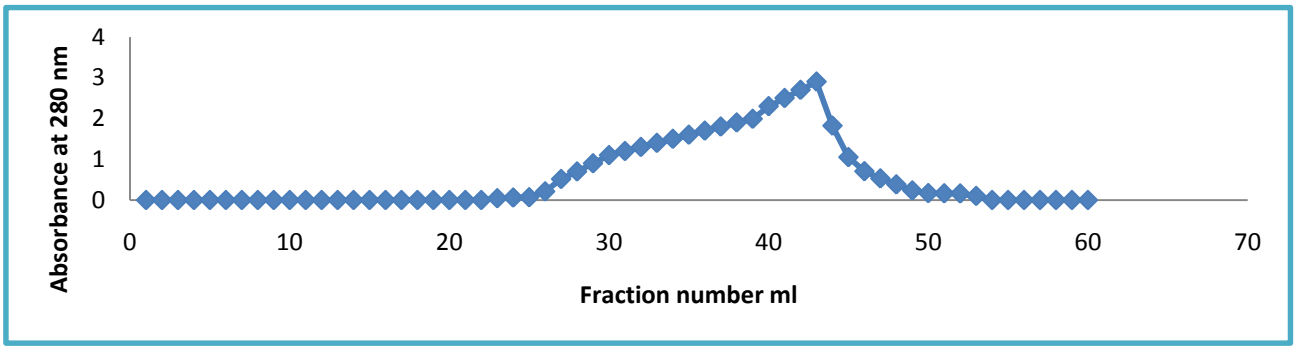

Figure 1: Purification of bacteriocin from isolate *Leuconostoc mesenteroides* ssp. *cremoris* on the column of the sphacryl S-200 gel and with a distance of 1.5 x 60 cm and a flow velocity of 18 ml / h by 3 ml / fraction. Recovery was achieved using a 0.5 molar phosphate buffer

Combined Effect of Nanoparticles and Bacteriocin against *L. monocytogenes*

The MIC of bacteriocin, silver and zinc nanoparticles was found to be 400 µg/ml against *L.monocytogenes* isolates. Antilisterial activity of *L. mesenteroides*ssp. *cremoris* bacteriocin and nanoparticles was tested by co incubation of *L.monocytogenes* with bacteriocin, silver nanoparticles, zinc nanoparticles, bacteriocin with silver nanoparticles and bacteriocin with zinc nanoparticles separately in soft cheese for 24h.

The results showed that 1ml of 400µg/ml of bacteriocin ,silver nanoparticles and zinc nanoparticles reduced the growth of *L.monocytogenes* in cheese with inhibition percent(49.48, 19 .38,38.14)% respectively. The combination between bacteriocin with nanoparticles (1:1) showed increased in the reduction of *L.monocytogenes* growth with (58.76 and 67.01) % for bacteriocin with silver nanoparticles and bacteriocin with zinc nanoparticles respectively (Figure 2). Some studies revealed that the *L. monocytogenes* growth was reduced in taleggio and milk in the presence of isolates which produce bacteriocin [25]. Mantovani and Russell [26] revealed that *L. monocytogenes* is sensitive to lactic acid bacteria bacteriocins .The supernatant of *Streptococcus thermophilus* have shown inhibitory effect against *L. monocytogenes* in soft cheese [27].

Naghmouchi *et al* [28].Showed the combination of Pediocin / Nisin was effective against *L. monocytogenes* .Aziz et *al* [29].Showed that the bacteriocin produced by *L. mesenteroide* sssp. *cremoriss* had inhibitory activity against pathogenic bacteria(*E. coli, Shigella group, Salmonella ser. paratyphi A* and *V. cholera*). Silver nanoparticles have been considered as protective agent that increase fruits half-life [30], and to reduce the coliforms contamination in cheese [31]. The results of Belluco *et al* [32] revealed that *L. monocytogenes* is susceptible to silver and that the effectiveness is related to ionic release.

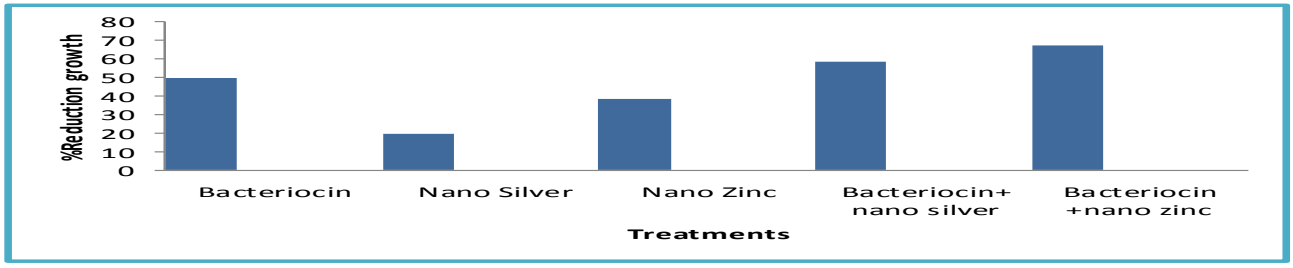

Figure2: Combined effect of bacteriocin and nanoparticles on *Listeria monocytogenes* growth in cheese

Conclusion

The results of current study concluded that *L. mesenteroides* ssp. *cremoris* bacteriocin and nanoparticles have a good activity against listeria, so it can be used as a protecting agent against *L. monocytogenes* in the soft cheese processing.

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