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Efficacy of alginate: Nanoliposome encapsulated pediocin against *L. innocua* in milk system

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Abstract
The present study was carried out to test the efficacy of liposome-alginate-guar gum encapsulated pediocin, a class-II bacteriocin against *L. innocua* in milk system. Initially, Ultra-turrax homogenizer was used to prepare a coarse emulsion of phospholipid in pediocin solution. Nanoliposomes prepared by high pressure homogenization showed a decrease in liposome size with increase in homogenization pressure and decrease in particle size distribution with increase in number of cycles. The hybrid capsules were compared for its efficacy to deliver pediocin in milk system. However, the encapsulation in alginate as well as in liposomes failed to enhance pediocin activity and inhibit the growth of *L. innocua* in milk system at both the tested temperatures. Hence, the efficacy of the developed microcapsules can change in different food matrices, which necessitates the microcapsules to be extensively studied for its release mechanics in the respective system before testing against target organisms.

Keywords: Microencapsulation, Bacteriocins, Pediocins, Liposomes, Biopreservation, Milk

1. Introduction
With the increased consciousness among the consumers regarding the quality and safety of food products, the use of chemical preservatives has really posed a big question. In this regard the use of bacteria having GRAS status or its metabolites comes as a suitable alternative. Bacteriocins are ribosomally-synthesized peptides or proteins with antimicrobial activity, produced by different groups of bacteria [1]. Some of the widely explored bacteriocins for food applications are that of lactic acid bacteria, a group of bacteria bearing the GRAS status. Pediocin is a small, heat stable, group II bacteriocin having a strong anti-listerial activity and broad range of activity. It is produced by the genus *Pediococcus* that are used commercially in meat and vegetable fermentations. Pediocin acts by permeabilizing the cell wall of target bacteria causing cell leakage and finally death of target bacteria [2].
The mode of application of bacteriocins in food system has traditionally relied on direct addition in food. However during prolonged storage of food products these bacteriocins may lose its stability and activity due to enzymatic degradation in food system [3]. The direct addition of higher dose of bacteriocins in food may also create problem of development of resistant target strains [4]. One of the potential methods to protect these antimicrobials from external food ingredients and increase its stability and efficacy is microencapsulation technology. Microencapsulation is a technology whereby the target molecule is packaged in miniature, sealed capsules to protect it from external factors and deliver in the targeted site under specific conditions [5]. The technology also helps to control the release of target molecule in a controlled rate.
Among the common encapsulating materials used in food system, the alginate system is blessed with a wide array of techno-functional attributes that makes it one of the most commonly used biopolymers in the food system [6]. It is basically a linear polysaccharide composed of variable proportions of β-D-mannuronic acid (M block) and α-L guluronic acid (G block) linked by 1–4 glycosidic bonds. In the presence of di- or tri- valent cations the alginate system forms stable hydrogel [3]. However the alginate matrices are macro porous structure with variable pore size which may leak out the low molecular weight biomolecules, resulting in low entrapment efficiency [7]. Hence the alginate system may require reinforcement with liposomes and other fillers. Typically liposomes are vesicular structures consisting of hydrated bilayers that form spontaneously when phospholipids are dispersed in water through external energy. Phospholipids are amphiphilic in nature; hence during the

dispersion in the aqueous solution the non-polar region orient themselves towards the interior away from the aqueous phase and the polar region towards the aqueous phase [8]. The commonly used methods for making liposomes are physical (membrane emulsification, sonication, high pressure homogenization, microfluidization and colloid mill) and chemical (reversed-phase evaporation and depletion of mixed detergent–lipid micelles) methods [3]. High pressure homogenization is a highly preferred technique due to its higher volume processibility, better reproducibility and its ability to form homogenous droplet size distribution [9]. The size of the liposomes has a direct impact on the stability, entrapment efficiency and the release rate of the entrapped molecule [10].

The effectiveness of nisin entrapped liposomes against *L. monocytogenes* was studied by da Silva *et al.,* [8] in milk system; although it was found that nisin loaded liposomes were less efficient in controlling *L. monocytogenes* growth, when compared to free nisin. However the hybrid alginate-liposome system when investigated by Dai *et al.,* [7] was found to be effective in controlled protein delivery. Therefore, very limited studies have been undertaken to check and validate the efficacy of liposome-polysaccharide system for controlled antimicrobial delivery in food system. Hence the present study was undertaken to study the efficiency of alginate-guar gum system containing pediocin loaded nanoliposomes to deliver pediocin at a controlled rate in milk system.

2. Materials and Methods

2.1 Materials

The bacteriocin, pediocin from *Pediococcus pentoseus* 34 strain was produced and processed as mentioned by Narsaiah *et al.,* [11]. Phosphotidylcholine (L-α-phosphatidylcholine) (PC) was obtained from Sigma-Aldrich, USA, Sodium alginate and guar gum from SD Fine Chemicals Limited, India, and MRS broth, brain–heart infusion (BHI) broth, tryptone glucose extract (TGE) agar and Skim Milk Powder (SMP) were purchased from Himedia Laboratories, India.

2.2 Preparation of Nanoliposomes

The nanoliposomes were prepared by two step process. Firstly, a coarse emulsion was made by adding 200 mg of PC to 50 ml of Pediocin solution. A rotor-stator system using Ultra-Turrax Digital homogenizer- probe S25N-25G (IKA T-25, USA) was first used to make the emulsion. The rotation per minute (rpm) of Ultra-Turrax Digital homogenizer was varied from 10,000 to 12,000 rpm and time for shear were tested at 8, 10 and 15 mins. The coarse emulsion was then immediately passed through a high pressure homogenizer (Constant Systems Limited, UK). The pressure of HPH were tested at 10, 15, 20 Kpsi and number of pass was maintained at 1–5 cycles. Each emulsion was carefully sampled and then immediately analyzed for droplet size distributions. The best combination based on particle size and distribution was selected for further experimentation on encapsulation.

2.3 Particle Size and Zeta potential Measurement

The particle size of the emulsions and the Zeta potential of the corresponding solution were analysed by dynamic light scattering using a nanoparticle size analyzer (Zietasizer Nanoseries, ZEN3600, Malvern Instruments, UK). The instrument contains a 4-mW He-Ne laser operating at 633 nm wavelength. The measurement was conducted at 173° detection angle at 25 °C.

2.4 Encapsulation of Pediocin using Alginate and Guar Gum

The encapsulation of Pediocin contained nanoliposomes was carried out by the method of Narsaiah *et al.,* [11]. Briefly, a homogenous solution containing 2% sodium alginate with 0.4% guar gum as filler material was prepared by continuous stirring in an overhead stirrer. The nanoliposomes were then added at the rate of 20% to alginate–guar gum solution and again stirred for homogenous mixing. The capsules from the mixture were prepared by conventional dripping method in 0.2 M $CaCl_2$ solution. The beads were periodically separated from the solution by a muslin cloth and washed with distilled water to remove excess $CaCl_2$. The washed beads were then dried and used for further analysis.

2.5 Encapsulation efficiency

The encapsulation efficiency of the method described above was calculated by estimating the pediocin content of alginate-guar gum solution before dripping into $CaCl_2$ bath solution and the pediocin content in the formed capsules. Lowry method for protein estimation was used to estimate the amount of pediocin. The microcapsules were drawn from $CaCl_2$ bath solution at different time intervals and unit weight of microcapsules were then broken and dissolved in phosphate buffer (0.2 M/7.4 pH). The solution was centrifuged at 10,000 rpm/30 min and the supernatant was used for estimation of pediocin content. Encapsulation efficiency (EE) was calculated using the following formula:

EE = (Pediocin content in capsules/pediocin in polymer solution) x 100%

2.6 Release of Pediocin from Na-Alginate beads in phosphate buffer (pH 6.7)

The periodic release of pediocin from alginate microcapsules was first checked in phosphate buffer at milk pH. Briefly, 50 ml of autoclaved phosphate buffer (pH 6.7) was taken and added with 6 gm of microcapsules drawn from the $CaCl_2$ bath solution at different time intervals and incubated at 37ºC. The capsules inoculated buffer was periodically sampled for pediocin content over the time of incubation. The periodically drawn samples were centrifuged at 2000×*g* for 10 min, and the supernatant was analyzed for pediocin content. The amount of released pediocin with time was calculated as follows:

Fractional release (%) = (amount of pediocin released from the beads in milk/actual amount of pediocin initially entrapped in the beads) x 100%

The values of periodic release were then correlated with the efficiency of encapsulation.

2.7 Growth inhibition of *Listeria innocua* in milk system

The efficacy of the developed pediocin loaded microcapsules was studied against the growth inhibition of *Listeria innocua*, a non-pathogenic member of *Listeria* genus. Prior to inoculation in milk, the test organism *Listeria innocua* was grown in BHI broth at 37ºC to achieve active growth. An aliquot of 100 µl of active *Listeria innocua* was inoculated in different sets of flasks containing 50 ml sterilized RSM (Reconstituted skim milk, 12%). Pediocin was added in three forms: a. unencapsulated free pediocin (1 ml = 60,000 IU), b. liposome encapsulated pediocin (1 ml) and c. pediocin in liposomes encapsulated in alginate plus guar gum (6 g of capsules = 60,000 IU). One flask of RSM without pediocin was kept as a control. The flasks were incubated at two

temperatures 4 ºC and 37 ºC and periodically sampled for *L. innocua* at 0, 4, 8, 12, 24, 36 and 48 h by serial dilution method.

3. Results and Discussion
3.1 Nanoliposomes production
3.1.1 Ultra-turrax homogenizer
For the preparation of coarse emulsion, Ultra-turrax homogenizer was first used. It is a convenient method for coarse emulsion preparation and many previous studies have also reported on the use of Ultra-turrax (UT) homogenizer for creating emulsions [12, 13]. It is evident from Fig. 1 that the mean particle size after 12,000 rpm/15 min was 199.8 nm and poly dispersibilty index (PdI) as 0.66 (Fig. 1). The mean particle size and the PdI influence the physical stability, solubility, biological performance, release rate, turbidity and chemical stability of emulsion [10]. Although the technique can generate fairly lower particle size, however the relatively higher PdI limited the further use of UT for liposome preparations. Even though, the increase in rpm caused reduction in particle size, however higher rpm lead to higher heat production due to higher shear force and foaming due to protein activity. Similar to the reports of Das and Chaudhury, [14], it was also observed here that the homogenization time did not have significant effect on the size but did affect the efficiency of size reduction.

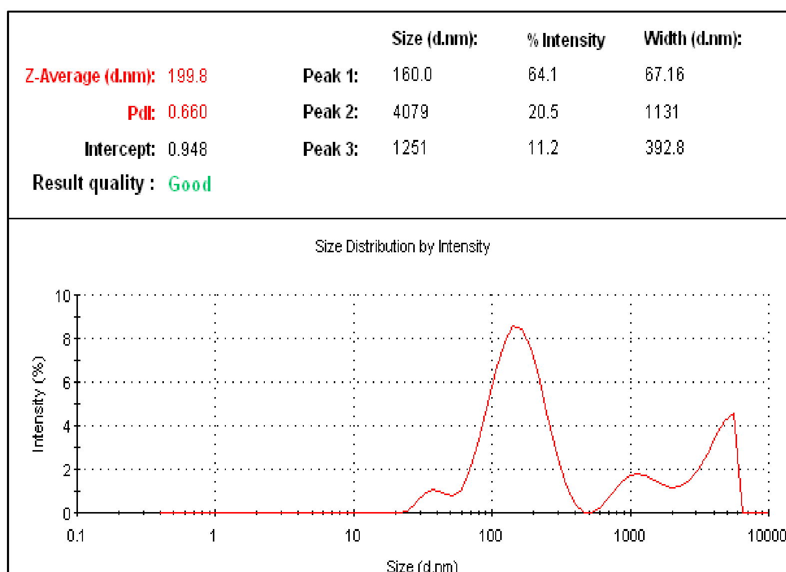

Fig 1: Ultra-Turrax homogenization at 12,000 rpm/15 mins

3.1.2. High Pressure Homogenization (HPH)
The pressure and the number of cycles in a High Pressure Homogenization (HPH) had significant influence on the properties of pediocin nanoemulsion. The pressure alters the shear forces and the turbulence that in turn alters the particle size and its distribution [15]. As shown in **table 1**, at 10,000 psi the increase in cycles progressively decreased the PdI or particle size distribution, however with not so significant decrease in particle size. The decrease in particle size vastly depended on the applied pressure. With the increase in homogenization pressure from 10,000 psi to 15,000 psi, after two cycles the mean particle size noted was 151.4 nm and PdI 0.275 (Fig. 2). Similar phenomenon on the effect of homogenization pressure and cycles on the mean particle size and the particle size distribution have also been reported by Yuan *et al.*, [16]. Based on these considerations, the combination of pressure of HPH at 15,000 psi and 2 cycles were selected for preparation of nanoliposomes in the further assays.

Table 1: Changes in Av. Size, PdI and Size-percentage with increasing cycles at 10,000 psi

Cycle	Av. Size(nm)	PdI	Size(nm) – percentage
First	236.6	0.698	184.6 - 59.7% 2829 - 38.5%
Second	241.1	0.668	315.6 - 77.8% 3895 - 22.2%
Third	182.4	0.375	214.6 - 88.8% 4169 - 11.2%
Fourth	178	0.403	253.2 - 91.9% 4369 - 8.1%
Fifth	159.6	0.347	221.5 - 94.7% 4013 - 5.3%

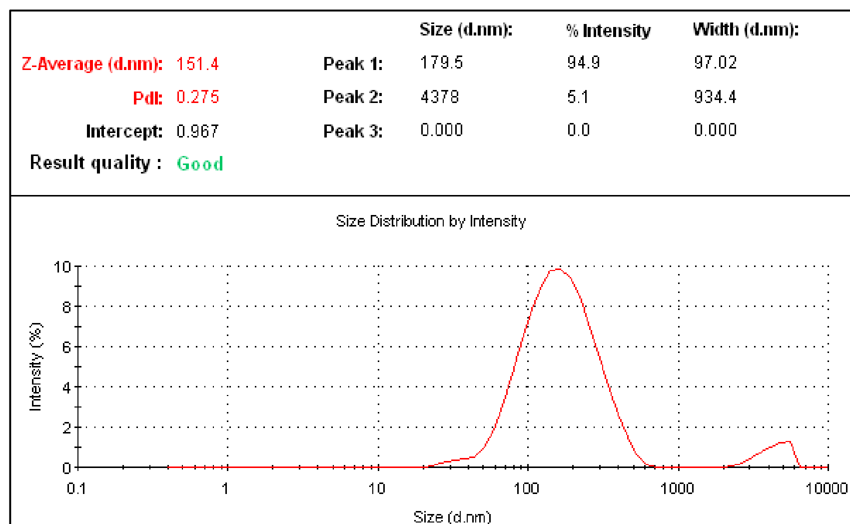

Fig 2: High pressure homogenization at 15,000 psi/2 cycles

3.1.3. Zeta potential

The Zeta potential measures the charge on the surface of the particle and indicates the physical stability of nanoliposome solution. For an electrostatically stabilized nanosuspension, a minimum zeta-potential of ± 30 mV is required to achieve a stable nanosuspension [10]. The average Zeta potential of the liposomal solution prepared by Ultra-Turrax homogenizer was -24.5 mV and that by high pressure homogenizer was -44.5mV. Hence, it is evident that the nanosuspension produced from HPH is more electrostatically stable than from Ultra-Turrax homogenizer. It was also observed that the zeta potential of the nanosuspension tended to vary with the addition or increase in protein concentration hence may require standardization with higher concentration of protein. The table below compares the differences in zeta potential with different method of liposome production and with changes in encapsulated biomolecule.

Table 2: Changes in Zeta potential with different method, wall material and biomolecule

Liposome[a]	Zeta potential (mV)	Method	Reference
PC-1 Nisin	−55.8	Thin-film hydration method	Malheiros *et al.* [17]
BLS P34	−27.42	Thin-film hydration method	Malheiros *et al.* [18]
PC-1-Cho Nisin	−64.2	Thin-film hydration method	Malheiros *et al.* [19]
BLS P34	−53.0	Thin-film hydration method	Malheiros *et al.* [19]
PC-1 Pediocin	-24.5	Ultra-turrax homogenizer	This work
Pediocin	-44.5	High pressure homogenizer	This work

[a]Liposomes prepared with phosphatidylcholine (PC-1) or PC-1-cholesterol (7:3)

3.2. Encapsulation of pediocin

Many previous studies have signified the importance of encapsulation of antimicrobial compounds in improving the activity through controlled release and protection from external food ingredients [8, 11]. In the same line of hypothesis, encapsulation of pediocin loaded nanoliposomes was carried out with a mixture of 2% Sodium-alginate and 0.4% guar gum as filler material.

3.2.1. Encapsulation efficiency versus bead hardening time

A study by Lee *et al.,* [20] carried out on the encapsulation of catechin with calcium pectinate reported a strong effect of bead hardening time in $CaCl_2$ bath solution on encapsulation efficiency. In this study also we tried to check the effect of bead hardening time in $CaCl_2$ bath solution on the encapsulation efficiency of the resulting alginate beads. The resulting alginate beads were also simultaneously analyzed for release rate of pediocin in phosphate buffer (pH 6.7). Table 3 shows that with the increase in bead hardening time from 2 mins to 32 mins, there was steady decrease of encapsulation efficiency from 70.06% to 21.57%. The increase in bead hardening time allows the calcium more time to penetrate the bead making the calcium-cross-linked network structure of the gel beads denser and compact with less void volume available for the entrapment of pediocin-loaded liposomes. Lee *et al.,* [20] also observed the same pattern of decrease in encapsulation efficiency with increased bead hardening time.

Table 3: Bead hardening time against Encapsulation Efficiency and Fractional release

Srl. No.	Time (min)	Encapsulation Efficiency (%)	Fractional Release (5 hr)%
1.	2	70.06	55.5
2.	12	40.52	45.28
3.	22	27.17	45.22
4.	32	21.57	34.93

3.2.3. Release behaviors in phosphate buffer

In order to select capsules with good encapsulation efficiency as well as good release rates for testing in the milk system against the test bacteria, *L. innocua,* the different sets of capsules having different encapsulation efficiency were tested in phosphate buffer (pH 6.7). Although the encapsulation efficiency was higher for capsules obtained after 2 min bead hardening time, it lost 55.5% of protein after 5 hours, hence capsules after 12 min bead hardening time were selected for testing in milk system since the protein release rate was comparatively better than the 2 min capsules and comparable to 22 min capsule. Additionally, the encapsulation efficiency

was better than the 22 min capsule (Table 3). Although, 32 min capsule had best release characteristics, its encapsulation efficiency was comparatively very low for testing in milk system. The variation in the observed fractional release can be related to the differences in calcium-cross-linked network structure of the capsules. More bead hardening time lead to more calcium cross linking and therefore stronger and denser network structure (however with lower encapsulation efficiency) and hence lower fractional release in phosphate buffer. At the same time the capsules containing increased encapsulated pediocin had relatively lower exposure time in $CaCl_2$ bath solution, hence the calcium cross-linking in the alginate beads may not be very extensive and strong enough to hold the pediocin for a relatively longer period of time, hence there was a rapid dissolution of pediocin when exposed in buffer. Our results are in accordance with an earlier report by Lee *et al.* [20], wherein similar observations were made.

3.3. Growth inhibition of *L. innocua* in milk system

Finally, the microcapsules exposed for 12 min in $CaCl_2$ bath solution was selected for testing in milk system based on the encapsulation efficiency and fractional release in phosphate buffer. Pediocin is a type II heat stable bacteriocin that are highly active against *Listeria* spp. *Listeria innocua* is a non-pathogenic bacteria of *Listeria* spp. that is very similar to the food borne bacteria *Listeria monocytogenes.* In this study, pediocin was tested against *Listeria innocua* in milk system in three different forms at two different temperatures. From the table 4, it is evident that at 4 ºC/48 hr, pediocin in free form inhibited *L. innocua* by 2 log cycles. However, no inhibition of *L. innocua* was observed with pediocin in liposomal or liposome-alginate hybrid matrix during the 48 hr of study. Similarly at 37 ºC, there was initial inhibition of 1 log cycle after 4 hrs by pediocin in free form, after which the growth of *L. innocua* increased and the number consistently increased to 8 log cycles after 48 hrs (table 5). However, the pediocin in liposomal or liposome-alginate hybrid matrix however failed to show any effect on *L. innocua* and the growth of *L. innocua* in these solution were similar to the control reaching to 9 log counts after 48 hrs of incubation.

Table 4: Influence of pediocin in different forms against the growth of *L. innocua* at 4 ºC

Time (hr)	Control	Pediocin	Liposome	Hybrid Capsule
0	10^6	10^6	10^6	10^6
4	10^6	10^4	10^6	10^6
8	10^6	10^4	10^6	10^6
12	10^6	10^4	10^6	10^6
24	10^6	10^4	10^6	10^6
36	10^6	10^4	10^6	10^6
48	10^6	10^4	10^6	10^6

Table 4: Influence of pediocin in different forms against the growth of *L. innocua* at 37 ºC

Time (hr)	Control	Pediocin	Liposome	Hybrid Capsule
0	10^6	10^6	10^6	10^6
4	10^7	10^5	10^6	10^7
8	10^7	10^6	10^7	10^7
12	10^8	10^7	10^7	10^7
24	10^8	10^8	10^8	10^8
36	10^9	10^8	10^9	10^9
48	10^9	10^8	10^9	10^9

Hence, the encapsulation of pediocin either in alginate system or in liposomal forms inhibited the pediocin activity instead of prolonging the pediocin action over the storage/incubation period at different temperature. da Silva *et al.,*[8] also witnessed a similar observation when testing liposomal nisin in milk system. Interaction of antimicrobial nisin with phospholipid fatty acid and milk fat were cited as major reasons. However in our case, free pediocin was able to inhibit the bacteria at both the temperature. Milk contains various minerals, major being Calcium and others (Phosphorous, Magnesium, Potassium, Sodium, Chloride etc.), hence these ions may interfere with the dissolution rates of the calcium-alginate beads which directly influence the release of contents from the beads. Hence the release behavior from the alginate beads as well as from liposomes can differ in different application platform, which needs to be first investigated before testing for antimicrobial action against different target bacteria.

4. Conclusion

The combined use of Ultra-Turrax homogenizer and High Pressure Homogenizer was successful in preparation of nanosize liposomes. The encapsulation efficiency of liposomal pediocin in alginate-guar gum matrix is inversely proportional to bead hardening time in $CaCl_2$ bath solution. The bead hardening time in $CaCl_2$ bath solution also affected the fractional release in phosphate buffer solution. The results from growth inhibition studies indicated that prior to testing the pediocin encapsulated capsules in milk, the alginate matrix needs to be extensively studied for its release mechanics in milk taking into consideration the ionic balance of milk system.

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