# The Utilization of Chitosan in *Streptococcus Agalactiae* Encapsulation as Mastitis Vaccine Carrier Candidate

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**Abstract.** Mastitis is a disease in dairy cattle that causes economic losses to farmers around the world. Mastitis is caused by microbes with *Streptococcus agalactiae* being the main cause of subclinical mastitis. The prevention and control of mastitis is through sanitation management, drug administration and vaccination programs. Administration of vaccines is one of the technical challenges posed by vaccination programs. To optimize vaccination, the development of oral vaccines with microparticle carrier systems can be pursued. The development of chitosan-based vaccine carriers for oral administration has garnered interest due to the substance's potential to boost immune response and its pharmaceutical properties. However, research into the development of vaccine carriers containing inactivated pathogenic bacteria to combat mastitis in dairy cattle is limited. In this study, *Streptococcus agalactiae* was encapsulated using chitosan-alginate microspheres through a simple poly ionic complexation method with high-speed centrifugation. This study aimed to determine the loading efficiency (LE) and capacity (LC) of *S. agalactiae* incorporated into chitosan microparticles as a mastitis vaccine carrier system. The results showed that bacterial encapsulation using chitosan complex at pH 5 resulted in LE and LC up to 87.1% and 2.4%, respectively. The addition of 0.022% tripolyphosphate (TPP) resulted in microparticles with higher LE and LC values. Fourier transform infrared (FTIR) investigation revealed a possible interaction between the two constituents of the microsphere *S. agalactiae* and the chitosan complex. These findings indicate that the chitosan complex loaded with inactive *S. agalactiae* has good potential as a vaccine carrier system for oral administration. Further characterization of microspheres is needed for their potential application in pre-clinical studies.

**Keywords:** chitosan · loading efficiency · loading capacity · mastitis · *Streptococcus agalactiae* · vaccine carrier


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## 1 Introduction

Mastitis cases in Indonesia are still one of the dominant diseases that cause economic losses, including decreased milk production, decreased milk quality, and increased medical costs [1]. Decreased production occurs in response to inflammation, both for clinical [2, 3] and subclinical mastitis [4]. The cause of mastitis is infectious microbes. Therefore, the best prevention is done by maintaining hygiene and sanitation of the cage [5]. Interventional mastitis cases are usually investigated separately for clinical and subclinical mastitis, although the two are sometimes recognized as correlated issues [6]. The strategy for treating mastitis is mostly antibiotics and treatment of subclinical cases in the infected quarters will lead to a reduction in the spread of clinical cases [7]. Prolonged exposure to certain levels of antibiotics can increase the risk of antibiotics resistance. Along with increasing concern for human health, it is necessary to develop alternative treatments and prevention of infectious agents [8]. A possible new alternative for the treatment of mastitis is bacteriocin [8], while the more comprehensive prevention and control of mastitis are realized through a vaccination program [9].

The development of vaccines against mastitis pathogens has advanced rapidly in the last decade. Currently, the mastitis vaccine available in the market is Startvac® (Hipra UK Ltd., Nottingham, UK), a polyvalent vaccine containing *E. coli* and several strains of *Staphylococcus aureus* which is applied by deep intramuscular injection [10]. There are a number of mastitis vaccines under experimental stage, most of which use *Staphylococcus aureus* and *E. coli* antigens with various routes of application including intramuscular, subcutaneous and intramammary [11]. Inoculation of vaccines by injection is the most commonly used method, but injection has several potential disadvantages, including pain, cross-contamination, needle stick injuries, under- or over-dose, and increased costs [12]. On the other hand, oral and nasal vaccine applications are still being used today, which offers several advantages such as: has much higher patient compliance; can be self-administered; does not need to follow strict sterile procedures; eliminates needle-associated risks; has greater capacity for mass immunizations and elicits antigen-specific sIgA/IgG providing a mucosal/systemic humoral immune response [13].

As a less invasive method, oral application also offers flexibility in the type of preparation to deliver the active substance to the target organ, one of which is the microencapsulation technique. Microencapsulation is an effective method in drug or vaccine delivery systems that allow for the storage of the active ingredient in a suitable form within the desired therapeutic concentration range, and optimizes the release of the active ingredient [14]. One of the microparticle materials that has received increasing attention is chitosan, which is a cationic polysaccharide product of chitin deacetylation and when combined with alginate (a product of algae extraction) is able to form ionic cross-links and improve the biological activity of the active ingredients it carries [16, 17]. Although numerous studies have been conducted on mastitis vaccines, oral delivery of mastitis vaccine ingredients using chitosan as a carrier has received less attention. This study aimed to determine the loading efficiency (LE) and capacity (LC) of the chitosan complex that entraps inactivated group B *Streptococcus* antigen (GBS-trehalose) as an oral subclinical mastitis vaccine candidate.

## 2 Materials and Methods

### 2.1 Materials

Low molecular weight chitosan with a degree of deacetylation of 75-85% was obtained from Sigma-Aldrich (CAS No. 9012-76-4). Group B *Streptococcus* (GBS) is characterized from *Streptococcus agalactiae* which were collected in previous studies [15]. Sodium alginate (food/halal-grade), sodium tripolyphosphate (TPP) and trehalose (pharmaceutical standard grade) were purchased from Sigma-Aldrich.

### 2.2 Methods

#### 2.2.1 Sample Preparation

The trehalose solution was prepared by dissolving 190 g of trehalose in 500 ml of phosphate buffered saline. Chitosan, alginate and TPP solutions were prepared in deionized water of 0.056%, 0.022% and 0.01-0.022%, respectively. The loading mixture was prepared by mixing 1 X $10^8$ cells/ml inactive GBS (adjusted according to McFarland standard) with 8-20 mg of trehalose solution, then stirred thoroughly using a vortex mixer (Maxi Mix II, Thermolyne, USA). In addition, the GBS-trehalose mixture was suspended in a chitosan solution and manually homogenized. Next, the GBS- chitosan complex was adjusted to pH 5.0 with 0.1 M HCl solution.

#### 2.2.2 Microparticles Preparation

Encapsulation is carried out by high-speed stirring and mixing, as recommended in the microencapsulation procedure with certain modifications [16]. The GBS-chitosan complex was added dropwise to the alginate solution whose pH was adjusted to 4 under stirring at 600 rpm. The complex was then added slowly with TPP solutions adjusted to pH 9. Then, the mixture was stirred at 600 rpm for 2 hours at room temperature, followed by centrifugation at 14000 rpm for 10 minutes to separate the unbound GBS.

#### 2.2.3 Loading Efficiency (LE) and Loading Capacity (LC)

Determination of LE and LC was carried out as described by Li et al. [17]. The loading efficiency and capacity of microparticle were detected indirectly by determining the unbound GBS collected in the supernatant after centrifugation as shown by the following procedure. One ml of the microparticle suspension was centrifuged (Centrifuge TGL-20 M, China) at 14000 rpm for 10 min and the amount of unbound GBS in the supernatant was measured using a UV-Vis spectrophotometer (Genesys-10S, Thermo Scientific, USA). Chitosan-alginate-TPP microparticle blank supernatant was adopted as a blank to correct the absorbance reading of the microparticles. The corrected optical density (OD) values were then used to calculate the free GBS concentration in the supernatant. The LE and LC values are then calculated according to the following formula:

$$\text{LE (\%)} = \frac{\text{total amount of GBS trehalose} - \text{unbound GBS trehalose}}{\text{total amount of GBS trehalose}} \text{ x } 100$$

**Table 1.** Microparticle formulation with chitosan-GBS complex as the constituent. Loading efficiency (LE)loading capacity (LC).

| Formula-tion | Chitosan % (w/v) | Chitosan pH | Alginate % (w/v) | Alginate pH | TPP % (w/v) | TPP pH | SGB (cells): Trehalose (mg) | LE % | LC % |
|---|---|---|---|---|---|---|---|---|---|
| F0 | 0.056 | 2.5 | 0.022 | 4.0 | - | - | 1 x $10^8$: 95 | 61.89 ± 5.37 | 1.39 ± 0.12 |
| F1 | 0.056 | 5.0 | 0.022 | 4.0 | - | - | 1 x $10^8$: 95 | 72.49 ± 4.98 | 1.56 ± 0.15 |
| F2 | 0.056 | 2.5 | 0.022 | 4.0 | 0.022 | 9.0 | 1 x $10^8$: 38 | 80.12 ± 2.12 | 2.26 ± 0.06 |
| F3 | 0.056 | 2.5 | 0.022 | 4.0 | 0.01 | 9.0 | 1 x $10^8$: 38 | 57.02 ± 4.6 | 1.6 ± 0.13 |
| F4 | 0.056 | 5.0 | 0.022 | 4.0 | 0.022 | 9.0 | 1 x $10^8$: 38 | 87.1 ± 2.5 | 2.45 ± 0.07 |

$$\text{LC}\,(\%) = \frac{\text{total amount of GBS trehalose} - \text{unbound GBS trehalose}}{\text{dried microparticle weight}} \text{ x } 100$$

### 2.2.4 Microparticles Characterization

Fourier transform infrared spectroscopy (FTIR) was used to identify the chemical structure of the microparticles and the possible interactions between its constituents. The FTIR analysis procedure was carried out as described by Anicuta et. *al* [18]. Prior to analysis, the chitosan-GBS microparticle mixture was freeze-dried using vacuum freeze dryer (Labconco, USA). The FTIR spectrum of microparticles was measured by IR Prestige-21 IR-Fourier spectrometer optical system (Shimadzu, Japan). The spectrum is 50 average scans are recorded at 4 $cm^{-1}$ resolution in the 450 to 4000 $cm^{-1}$ range using TGS detector.

## 3 Results

Concerning antigen encapsulation, loading efficiency (LE) is defined as the ratio of the number of antigen complexes in the microparticles to the total number antigen applied in microparticle formulation, while loading capacity (LC) is defined as the ratio of weight of antigen enclosed in the polymer phase to the polymer weight [19, 20]. As shown in Table 1, five formulations of chitosan complex and GBS-trehalose with various TPP and trehalose arrangements and pH treatments were evaluated in this study. Treatment of pH 2.5 and pH 5.0 was given to chitosan to determine the effect of differences in the solubility of the carrier substance on the LE and LC values. The use of 8 mg and 20 mg trehalose solutions in GBS was used to determine the effect of adding cryoprotectants to both LE and LC as well as the interrelationships between the constituent matrices.

Setting pH 2.5 on chitosan resulted in LE values of 57.02%-80.12% and 1.39%-2.26% LC, while at pH 5.0 higher LE and LC values were obtained with 72.49%-87.1%

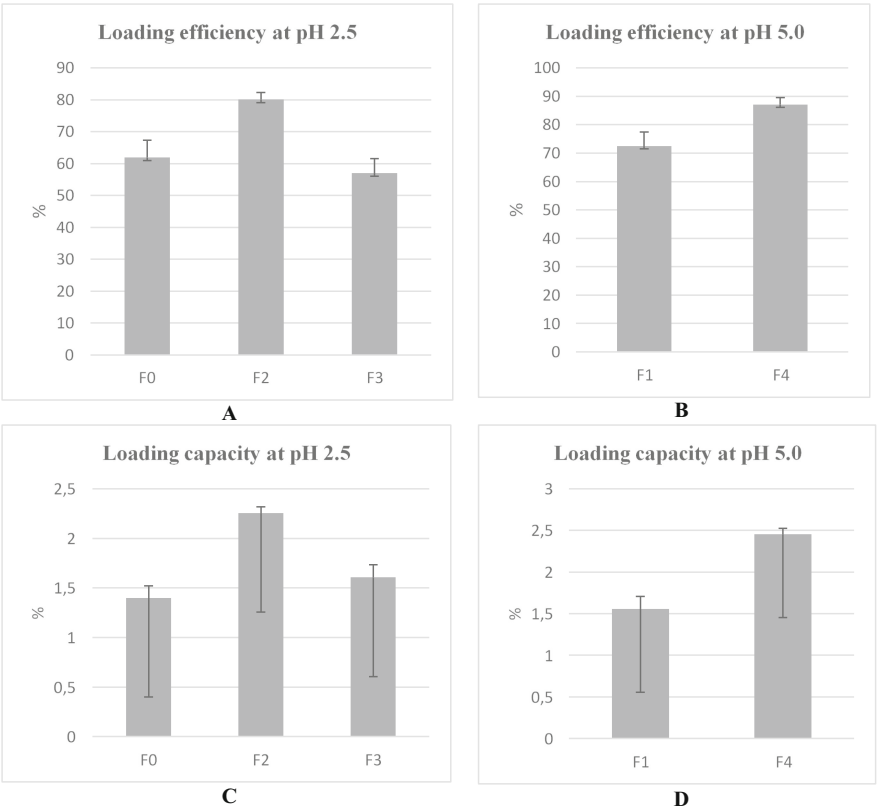


**Fig. 1.** Loading efficiency (LE) and LC values of microparticles based on pH treatment of chitosan as encapsulation material. Results are presented as mean ± SD (n ≥ 3).

and 1.56%-2.45%, respectively (Fig. 1 and Table 1). The percentage of LE and LC values was observed to be higher in microparticles using TPP when compared to without TPP, and this occurred especially in microparticles containing 0.022% TPP. The interaction of GBS with trehalose in smaller amounts (8 mg) resulted in microparticles with LE and LC values which tended to be higher, except for F3 with a lower pH of chitosan, which had an LE only 57.02%.

Figure 2 presents the FTIR spectrum which contains information on the molecular structure of chemical compounds and its use for characterization of chitosan-GBS microparticles. Experiments on the F4 formula resulted in LE and LC with the highest percentages so further investigations were carried out through FTIR. As shown above, the FTIR spectra has identified several bands in the 500-1645 $cm^{-1}$ region, whereas in the longer region (1660-4000 $cm^{-1}$) only a few bands were found but with slightly higher intensity. Several bands were observed to be overlapping in the region range of 500-804 $cm^{-1}$, 1152-1413 $cm^{-1}$ and 1624-1660 $cm^{-1}$. On the other hand, there is a broad band indicating the stretching vibration observed at 3315-3589 $cm^{-1}$.

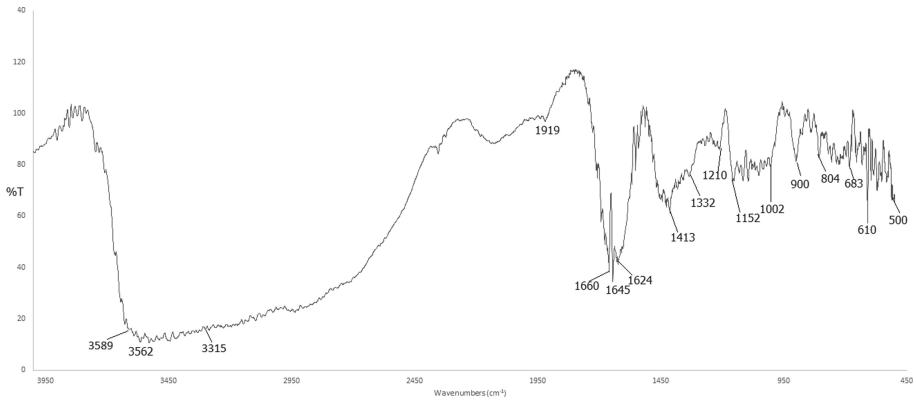


**Fig. 2.** Spectra for all components was used to obtain microparticles at F4 as a representation of other formulations containing chitosan-GBS complexes.

## 4 Discussion

From the findings above, there are indications of the effect of trehalose and TPP concentrations as well as the pH level of chitosan on the LE and LC values of microparticles. The efficiency value of 87.1% represents the proportion of GBS cells encapsulated within alginate beads coated with chitosan. Using chitosan with a higher pH (5.0) results in greater efficiency. The pH showed an effect on the binding of the chitosan complex to the alginate gel beads into a stronger complex formation, mainly by increasing the pH from acid to 5 or 6, this is because the pH is close to $pK_a$, value for chitosan, the charge density of the chitosan molecules will be reduced significantly, leading to a less elongated molecule with a higher diffusion coefficient [21, 22, 23]. This is in line with Gierszewska [25], that chitosan swelled and disintegrated rapidly in an acidic solution. Otherwise, chitosan-alginate membrane more able to withstand dissolution and stable at pH $\geq$ 5.5. This confirms that the chitosan solution at pH 2.5 produces lower efficiency when compared to pH 5.0. With TPP concentration of 0.022%, F2 and F4 were observed to have higher encapsulation efficiency. According to Anal and Stevens [24], it can be explained that TPP is a complexation agent that acts as a cross linker and strengthens chitosan-alginate microspheres. As a cross linker agent, TPP has negatively charged ions through ionic bonds with positively charged amino groups of chitosan to form chitosan beads [26]. Chitosan-alginate cross-linking with TPP affects the molecular and supermolecular structure, roughness and swelling behavior [25]. This leads to the strengthening of the GBS cells entrapment within it. The use of trehalose in this study was to coat GBS cells and protect them from the toxic effects of chitosan as well as a cryoprotectant. From the above observations, the use of high concentrations of trehalose, resulted in lower efficiency and capacity of GBS antigen. These results are in accordance with previous studies, that GBS coated with trehalose at the lowest concentration and entrapped in the soy-alginate protein isolate microcomplex obtained the highest LE and LC [15]. The involvement of trehalose leads to an antigen protection function rather than strengthening the microparticle structure. Thus, the use of trehalose must be limited to a certain concentration.

Characterization of chitosan-GBS microparticles via FTIR as shown in Figure 2 revealed a band extending from 3315-3589 $cm^{-1}$. The finding is in line with Sankalia et. al [27], that in general chitosan-alginate particles occur in the region band about 3500-3100 $cm^{-1}$ associated with improved hydrogen bonding compared to it from chitosan or sodium alginate only. In this band range, chitosan is associated with stretching vibrations of the O-H and N-H -OH, -CH2OH and -NH2 groups involved in hydrogen bonding, while in alginate the O-H stretching vibrations of the hydroxyl group are also involved in hydrogen bonding [25, 28]. In the spectra involving chitosan and alginate, non-acrylated 2-aminoglucose of chitosan usually occurs in the 1570 $cm^{-1}$ band along with symmetric and a symmetric -C-O of alginate at 1407 $cm^{-1}$ [27, 29]. These bands are not found in the spectra as shown in Figure 2. According to Honary at. al [30], the absence of these bands indicates the formation of electrostatic bonds between chitosan and alginate. Spectral vibrations in the 900-1800 $cm^{-1}$ region is considered to be a fingerprint of bacteria, cells and tissues which are mainly composed of lipids, proteins, nucleic acids and phosphates [31]. Prior to FTIR measurement, GBS-trehalose which was incorporated into the chitosan microcomplex was freeze-dried first. The effect of freeze-drying on a cell protein can be monitored by observing changes in the peak frequency of amide I (1500 $cm^{-1}$) and amide II (1700 $cm^{-1}$) [32]. Without the use of cryoprotectants, freeze-dried bacteria would normally experience a shifting down in the frequency of the observed wave numbers for amide I and amide II, reflecting a change in the bacterial protein structure [32]. In GBS-trehalose, the vibrational frequencies of amide I and amide II appear at around 1566 $cm^{-1}$ and 1624-1660 $cm^{-1}$, respectively (Figure 2). The shifting down in the frequency of amides I and II cannot be ascertained, because other formulations are needed for comparison. The addition of trehalose to bacterial cells can prevent changes in the bacterial protein structure [32]. It can be explained that trehalose protects the membrane by maintaining the lipid in the liquid crystal phase at the room temperature and may also suppress the phase transition in dry phospholipids by hydrogen bonds to the polar headgroups [33].

Recent research has shown that the microparticle formulation and pH have a significant impact on the performance of efficiency and capacity values. Chitosan-GBS microparticles exhibit encapsulation efficiencies of up to 87.1% and the potential for strong interactions between the constituent materials. These results suggest that the chitosan complex has the potential to be utilized as a vaccine antigen carrier, particularly for an oral mastitis vaccine. Further studies are needed to determine the ability to release antigen, microparticle characteristics, mucoadhesive, storage ability and toxicity.

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