Running title: Application of Sodium Alginate in Probiotic Orange Juice Microbeads

**Optimization of Sodium Alginate Level in Manufacturing Probiotic Orange Microbeads**

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*Received \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_; Accepted \_\_\_\_\_\_\_\_\_\_\_\_\_\_; Published \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_*

# Novelty statement

Probiotic functional foods are generally found in fermented and milk-based products. However, acceptance of this product is still limited, due to aftertaste, lactose intolerance, and milk protein allergies. This research is an innovation in the development of non-fermented and non-milk-based probiotic products, with the application of molecular gastronomy, namely using alginate-based microencapsulation technology with extrusion techniques. This research is meeting with the scope of diversified agriculture products for industrial use.

**Abstract**

Probiotic foods are common in the form of dairy-fermented food, which is limited in its acceptance due to the aftertaste, lactose intolerance issue, and milk-protein allergies. In addition, molecular gastronomy, a scientific discipline related to food processing based on food science is gaining more interest, and one of the implemented food sciences is alginate-based-microencapsulation with extrusion method. This research aims to optimize the level of sodium alginate (1%, 1.5%, 2%, 2.5% (w/v)) in the production of probiotic orange microbeads as non-dairy and non-fermented probiotic products. The optimization is based on the microbead’s morphology analysis (area, circularity, roundness, solidity) using the Fiji Image-J application, encapsulation efficiency (%EE) calculation according to total Lactic Acid Bacteria (LAB) comparison, yield, and pH of microbeads. The results show that the elevation level of sodium alginate significantly affects the morphology, EE, and yield of probiotic orange microbeads. The size and shape of the microbeads increase as the level increases sodium alginate. The maximum encapsulation efficiency (EE) and yield are achieved with sodium alginate concentrations of 1.5% and 2.5% (w/v), respectively, yielding 84.60% ± 5.03% for EE and 78.38% ± 0.92% for the yield. The optimal sodium alginate level is at a concentration of 2.5%, considering the most favorable form and morphology of microbeads, along with a total LAB meeting the minimum standards for probiotic product viability, which is 7.32 ± 0.19 log CFU/g.

**Keywords**: Encapsulation; Microbeads; Morphology; Orange juice; Probiotics

**Introduction**

The term "probiotic" comes from the Greek, namely "pro" which means to promote" and "biotic" which means life (Manzoor et al., 2022). According to the Food and Agriculture Organization (FAO), probiotics are microorganisms that can grow naturally in the intestines and when given in sufficient quantities can have beneficial effects on human health. Probiotic bacteria are useful for improving health if the culture is consumed alive, in sufficient quantities, can survive in the digestive tract, and survive during processing and storage (Jackson et al., 2019). Probiotics are often found in fermented foods such as yogurt and cheese. However, its acceptance is limited to certain groups due to the cause of aftertaste and current consumer preferences for non-dairy products due to awareness of the shortcomings associated with the intake of dairy products, such as lactose intolerance, high cholesterol content, and milk protein allergies (Aspri et al., 2020). This opens up opportunities to diversify other probiotic products that can be accepted by the wider community by adding probiotics to orange juice.

 Oranges are good carriers of probiotic cells because they contain high levels of vitamins, minerals, dietary fiber, and antioxidants (De Prisco & Mauriello, 2016). The viability value of probiotics in food ranges between 6 - 7 log CFU/g or /mL for efficacy and colonization on the surface of the colon. Therefore, efforts are needed to protect the viability and stability of probiotics from intrinsic (pH, water activity, oxygen molecules, food composition, added food additives, and oxidation-reduction potential) and extrinsic factors (temperature, relative humidity, and gas composition). It is possible to damage probiotic cells during preparation, processing, or production, as well as during long storage periods. Therefore, a non-fermentation preservation method is needed that can maintain the viability of probiotic bacteria, namely the microencapsulation technique. Microencapsulation is a technology of coating or layering the core substance with a polymer wall layer to protect sensitive components (Kamil et al., 2020). Microencapsulation has been developed in a scientific discipline related to food processing processes which is based on food science, namely the field of molecular gastronomy.

 Microbeads is an application of molecular gastronomy based on microencapsulation technology with alginate-based extrusion techniques. The protective agent used to protect the core material is sodium alginate because it is harmless, easy to produce strong beads, has good gelling and thickening properties, and is biocompatible and biodegradable (Cao et al., 2020). Sodium alginate is a biopolymer produced from extracts of various brown seaweeds and two genera of bacteria (*Pseudomonas* and *Azotobacter*) so it is abundant and relatively cheap (Li et al., 2024). Probiotic microencapsulation must meet criteria including the type of probiotic strain (core), microencapsulation material (coating), mechanism and characteristics of probiotic release as well as the appropriate microencapsulation method. The amount of coating material, in this case alginate, can certainly influence the microbeads produced. Therefore, there needs to be research on optimizing sodium alginate levels to produce probiotic orange juice microbeads. This research aims to optimize the level of sodium alginate in the production of probiotic orange microbeads as non-dairy and non-fermented probiotic products, observed from its morphology, EE, yield, and pH.

**Material and Methods**

**Raw materials used in this research**

Orange juice was produced from orange fruits with a variety of Medan (*Citrus sinensis* L.), purchased from a local market in Banyumanik, Semarang. Probiotic powder (*Lactobacillus acidophilus*, *Bifidobacterium longum*, and *Streptococcus thermophilus*) (Lacto-B, Indonesia), sodium alginate (JRS, Germany), calcium lactate (Galactic, Belgium), and de-Man Rogosa Sharpe Agar (MRSA) (Merck, Germany).

**Production of probiotic orange microbeads**

Probiotic orange microbeads were produced using an extrusion technique, according to Naklong *et al.* (2023) with modification. Briefly, 25 mL pasteurized orange juice (80°C ± 3.5 minutes) was mixed with 1 g of probiotic powder (Lacto-B) with initial viability in the range of 1 x 109 CFU/g, and sodium alginate (1%, 1.5%, 2%, and 2.5% w/v). The mixture was then dripped with a 20 mL syringe (OneMed, Indonesia) into 3% (w/v) calcium lactate solution, followed by hardening time for 30 minutes. The drip distance was set at 10 cm from the surface of the calcium lactate solution.

**Morphological analysis of microbeads**

Morphological analysis of probiotic orange microbeadswas performed byusing the Fiji Image-J application, referring to Martinović *et al.* (2023) with modification. Ten microbeads were randomly picked, placed on a black background separately, and captured with a camera. The resulting photo was analyzed for area, circularity, roundness, and solidity by using the Fiji Image-J application.

**Calculation of Total LAB and EE**

Total LAB was analyzed by using the plate count method, referring to Mohammadkhani *et al.* (2023). One gram of the sample was diluted in 9 mL sterile 0.85% NaCl. At appropriate dilution, 1 mL of bacterial suspension was transferred into the Petri disk and poured with MRS medium, after that, the Petri disk was incubated at 37°C for 48 hours. EE analysis was calculated by comparing the total LAB after and before the microencapsulation process, as follows:

%EE = $\frac{Total LAB after encapsulation (Log CFU/g)}{ Total LAB before encapsulation (Log CFU/g)}$ × 100

**Yield Microbeads (%)**

The analysis was carried out by calculating the comparison between the weights of microbeads produced with the weight of the initial material which includes the weight of orange juice that has been added with sodium alginate and probiotic powder Lacto-B (Ergin et al., 2021). The formula for calculating yield is as follows:

Total Yield (%) = $\frac{weight of microbeads (g)}{Weight of material (g)}$ × 100

**pH Value Analysis**

The pH value of probiotic orange microbeads was tested using pH meters (ATC, China), which were initially calibrated with buffer pH 4, and 7 (Siekkinen et al., 2023). Before the analysis, the probiotic orange microbeads were mashed, and the leaked juice was used for the pH analysis.

**Data analysis**

The obtained data was statistically analyzed with SPPS for the Windows 26.0 application. Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) was performed to analyze any significant effects of a given treatment. Analysis was performed at a significant level of 5%.

**Results**

**Morphological of probiotic orange microbeads**

The morphology of probiotic orange microbeads in terms of area, circularity, roundness, and solidity can be seen in Table 1. In addition, Figure 1 represents the probiotic orange microbeads for each treatment. Table 1 shows that different levels of sodium alginate improve the morphology of the microbeads significantly. The highest area, circularity, roundness, and solidity values obtained ​​at the 2.5% sodium alginate level, which were 17.91 ± 5.83 mm2, 0.52 ± 0.15, 0.61 ± 0.02, and 0.60 ± 0.18, respectively. Probiotic orange microbeads at a sodium alginate level of 2.5% produce regular and round sizes and shapes of microbeads, do not break easily, have a denser (springy) texture, produce more liquid when pressed, have a firm surface, and do not stick to each other.

**Total LAB and EE of probiotic orange microbeads**

Table 2 represents the total BAL and EE of probiotic orange microbeads. According to Table 2, it can be seen that the addition of a level of sodium alginate significantly increases the total LAB and EE of microbeads at 1% – 1.5% but experiences a decrease at alginate concentrations of 2% and 2.5% (w/w). Besides, the total LAB in all sodium alginate concentrations is still per a minimal dose of probiotic for its efficacy, which is log 6 -7 CFU/g.

**Yield of probiotic orange microbeads**

The yield of probiotic orange microbeads can be seen in Table 3. The increment of sodium alginate concentration significantly increases the yield of microbeads. The highest yield was obtained in microbeads with a 2.5% sodium alginate concentration, which was 78.38 ± 0.92.

**pH of probiotic orange microbeads**

The pH value is a measure of the acidity or alkalinity of a solution to assess the stability of the sodium alginate used in microbead production. pH value of microbeads with different levels of sodium alginate can be seen in Table 4. There is no significant difference in pH value caused by levels of sodium alginate. However, the pH value tends to increase.

**Discussion**

Morphological analysis of microbeads is a parameter for knowing the characteristics including the size and shape of microbeads. Circularity is an indicator to measure the degree of conformity to a perfect circle with a value ranging from 0 to1. A value of 1 represents a perfect circle shape (Moradi Pour et al., 2024). Roundness is an indicator to measure the curvature of the edges and corners of microbeads and solidity is an indicator to measure the compactness and fineness of particles with a value in the range of 0 to 1. The size and shape of the microbeads increase as the sodium alginate increases. This is due to the increase in polymer solution and its effect on viscosity. Increasing alginate concentration has been proven to increase the viscosity of the solution thereby producing a more stable size of microbeads (Seth et al., 2017). According to Julaeha *et al.* (2023) the addition of sodium alginate can increase the viscosity and mechanical strength of a mixture, which results in stronger granules.

 In addition, the number of cross-link interactions between sodium alginate and Ca2+ ions can produce a strong bead structure and can withstand the pressure of substance diffusion active into solution (Kalalo et al., 2022). The size, shape, and mechanical stability of gel particles can vary depending on the nature of the polymer matrix composition. For example, increasing the concentration of polymer and Ca2+ can cause the mechanical properties of the gel to increase (Sánchez-González, 2021). In addition, the shape and size of microbeads are influenced by several factors such as the number of free ions, viscosity, the concentration of polymer solution, distance between needles, hardening time, needle size, and surface tension (Ćujić et al., 2016). Nevertheless, the size of the microcapsules doesn't directly impact how efficiently materials are encapsulated. Instead, it plays a role in the controlled release of active ingredients, alongside the physical and chemical characteristics of the encapsulating material. This is because smaller microcapsules offer a larger surface area and increased contact with enzymes or release media, resulting in a more substantial release.

Microbeads with a level of sodium alginate of 2.5% produce a round shape in comparison to microbeads with other treatments. Microbeads with a 2.5% sodium alginate level have a regular and round shape, are not easily crushed, the texture is denser (very chewy), produce more liquid when pressed, the surface is firm, and do not stick to each other. The higher the sodium alginates, the harder the microbeads’ texture, because it can retain a significant amount of liquid (Abdin et al., 2021; Ćorković et al., 2021; Martinović et al., 2023).

Sodium alginate can transform into a hydrogel when it encounters divalent cations such as Ca2+ and contains over 98% water within the hydrogel (Mohammadkhani et al., 2023). The structural strength of the produced microbeads is enhanced when using a high concentration of alginate solution with high viscosity. Increasing the concentration of sodium alginate induces a higher level of cross-linking between gel networks, resulting in a denser and stiffer gel structure. According to Voo *et al.* (2016), the structural strength of the resulting microbeads is stronger when using high levels of sodium alginate. Elevated levels of sodium alginate induce association levels of higher cross-linking between gel networks resulting in the structure of a denser and stiffer gel (Karim et al., 2016; Tanganurat, 2020). The irregular surface shape of microbeads can be caused by intrinsic physical properties, such as surface tension and viscosity (Akram et al., 2019).

 The increase in EE of microbeads at 1% – 1.5% but experiences a decrease at alginate concentrations of 2% and 2.5% (w/w) is because of the complexity of the cross-linking structure, as observed in research by Mohammadalinejhad *et al.* (2023). EE also depends on the viscosity and Ca2+ diffusion through the matrix towards the binding sites. This aligns with the research of Tzatsi and Goula (2021) stating that the space occupied by sodium alginate aims to reduce the free volume within the sodium alginate matrix, creating a denser structure with smaller pore sizes. As a result, fewer core materials get trapped in the pores. Microbeads formed with a high level of sodium alginate tend to experience a decrease in porosity, preventing trapped substances from being released (Dehghannya et al., 2018).

Effective encapsulation is characterized by the trapping of numerous bioactive components, resulting in high EE and demonstrating the precision of the applied technology (Timilsena et al., 2020). EE is influenced by various parameters such as matrix porosity, hydrophilicity, wall material concentration, and physicochemical interactions between encapsulating components and carrier substances. The total LAB calculated with sodium alginate levels of 1% to 2.5% has met the sufficiency of probiotics in microbeads as it aligns with the recommended minimum efficacy dose of probiotics, which is 6-7 log CFU/g (Ding et al., 2022).

Yield is a fundamental parameter required during the production, packaging, and storage processes. The increase in yield is influenced by the natural characteristics of sodium alginate, which produces sponge microcapsules with more retained water. Additionally, it is influenced by the higher weight of the coating material compared to the sample. This is consistent with previous research stating that the higher sodium alginate used, resulted in higher yield (Muchsri et al., 2015).

The pH values obtained from different levels of sodium alginate are not significant, although showing an increment trend. Microbeads with a 2,5% sodium alginate concentration exhibit a higher pH value compared to other treatments due to the increased concentration of alginate added to the orange juice. The rise in pH is attributed to the commercial sodium alginate having a pH of 5.52 at room temperature. Generally, the pH of alginate ranges from 3,5 – 10 (Kamisyah et al., 2023). Therefore, a higher alginate concentration can elevate the pH values of the microbeads. The pH value in microbeads is a crucial parameter as it can affect other parameters. The stability of sodium alginate affects the pH value, and vice versa. A very low pH can reduce the stability of sodium alginate, thereby decreasing the viscosity of the solution. Reduced solution viscosity can result in smaller sizes and shapes of the produced microbeads.

**Conclusion**

Based on the research findings, it can be concluded that the sodium alginate level significantly enhances the morphology (area, circularity, roundness, and solidity), EE, and yield of microbeads. However, it does not affect the pH value. The 2.5% sodium alginate level is the optimal amount for the tested parameters, even though the highest encapsulation efficiency is achieved at the 1.5% sodium alginate level. Nonetheless, the encapsulation efficiency at the 2.5% sodium alginate level also results in a total BAL that meets the minimum standard for probiotic dosage.

**Acknowledgments**

This research was funded by LPPM Universitas Diponegoro, program scheme of Riset Pengembangan dan Penerapan (RPP) 2023.

**Author contributions**

RZK: Conceptualization, research design, methodology, validation, writing – reviewing, and editing; SBA: validation, writing – reviewing, and editing; STD: Conceptualization, validation, writing – reviewing, and editing; NFH: Investigation, data collection, formal analysis, writing – original draft.

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**Fig. 1**: Visualization of probiotic orange microbeads at different levels of sodium alginate

**Table 1:** Morphological Analysis of Microbeads

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Sodium Alginate (w/v) | Area (mm2) | Circularity | Roundness | Solidity |
| 1.0 | 7.80 ± 1.66 a | 0.24 ± 0.06 a | 0.44 ± 0.04 a | 0.35 ± 0.06 a |
| 1.5 | 9.17 ± 1.17 a | 0.26 ± 0.04 ab | 0.52 ± 0.02 b | 0.38 ± 0.02 a |
| 2.0 | 11.79 ± 4.97a | 0.38 ± 0.16 c | 0.59 ± 0.02 c | 0.42 ± 0.17 a |
| 2.5 | 17.91 ± 5.83c | 0.52 ± 0.15 c | 0.61 ± 0.02 c | 0.60 ± 0.18 c |

Data are shown as the mean ± standard deviation of 5 replications. Different superscript in the same column shows significant differences (p<0.05) based on ANOVA and DMRT.

**Table 2:** Total LAB and EE of probiotic orange microbeads

|  |  |  |
| --- | --- | --- |
| Sodium Alginate (w/v) | Total LAB (log CFU/g) | EE (%) |
| Before | After |
| 1.0 | 9.43 | 7.52 ± 0.25 a | 79.80 ± 2.59 a |
| 1.5 | 7.98 ± 0.45 b | 84.60 ± 5.03 b |
| 2.0 | 7.38 ± 0.10 a | 78.20 ± 1.30 a |
| 2.5 | 7.32 ± 0.19 a | 77.80 ± 1.79 a |

Data are shown as the mean ± standard deviation of 5 replications. Different superscript in the same column shows significant differences (p<0.05) based on ANOVA and DMRT.

**Table 3:** Yield of probiotic orange microbeads

|  |  |
| --- | --- |
| Sodium Alginate (w/v) | pH value |
| 1.0 | 44.64 ± 4.31 a |
| 1.5 | 53.03 ± 5.24 a |
| 2.0 | 62.10 ± 3.04 b |
| 2.5 | 78.38 ± 0.92 c |

Data are shown as the mean ± standard deviation of 5 replications. Different superscript in the same column shows significant differences (p<0.05) based on ANOVA and DMRT.

**Table 4:** pH of probiotic orange microbeads

|  |  |
| --- | --- |
| Sodium Alginate (w/v) | pH value |
| 1.0 | 5.79 ± 0.14 |
| 1.5 | 5.81 ± 0.15 |
| 2.0 | 5.91 ± 0.15 |
| 2.5 | 5.97 ± 0.09 |

Data are shown as the mean ± standard deviation of 5 replications. Different superscript in the same column shows significant differences (p<0.05) based on ANOVA and DMRT.