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Increasing Viability of *Bacillus subtilis* BR610 through Inulin-Loaded Synbiotic Microcapsules

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***Abstract*—This study aims to determine the growth pattern of Bacillus subtilis BR610 isolated from the intestines of Rabbitfish on broth + inulin nutrient media, the diameter of beads, encapsulation rate, absorption efficiency, and probiotic viability in synbiotic microcapsules when exposed to simulated bile and high temperature. The study was designed with a completely randomized plan. The treatments tested were inulin and alginate concentration, viability in 10% bile, temperatures of 70oC and 90oC. An overview of granular synbiotic microcapsules is presented in the form of images, while the quantitative data obtained was processed using ANOVA with the help of the SPSS application. *BR610* synbiotic microcapsule beads are round to oval in shape, transparent white in color, and the granules' elasticity increases with increasing alginate concentration. Statistical test results showed that 1% inulin significantly increased the population of *B. subtilis BR610*, a diameter of beads 0.9-3 mm, viability of probiotics in beads was 7.776 ± 0.06 log CFU/mL. The highest rate and efficiency of encapsulation and survival of probiotics on simulated were obtained from beads with 2% alginate and 1% inulin concentration. Synbiotic microcapsules can protect probiotics from environmental stress. This research serves as a scholarly resource on the utilization of probiotics in diverse domains, including fish feed production. It is well-established that the temperature within the feed molding apparatus can exceed 70oC during the fabrication of fish feed pellets. However, this is no longer a hindrance due to the implementation of alginate coating on probiotics.**

***Keywords*—Alginate; Bacillus subtilis BR610; extruded; inulin; Rabbitfish.**

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I. INTRODUCTION

Diversification of cultivated fish is expected to provide food needs. Rabbitfish is one type of marine fish that can be cultivated because it is adaptable to the cultivation environment [1], [2]. Naturally, Rabbitfish has a habit of eating seaweed or algae around it [3], [4], so the innovation of adding synbiotics to fish feed formulations is expected to help the process of nutrient absorption in the fish intestine become more efficient.

Synbiotics are a combination of probiotics and prebiotics [5]. Adding prebiotics in synbiotic preparations is expected to support probiotic nutrient needs. Prebiotics are substrates selectively used by host microorganisms that provide health benefits. Generally, prebiotics is derived from oligosaccharides and crude fiber. Some types of oligosaccharides that have been reported to be used as prebiotics are Chito-Oligosaccharide/COS [6]. Apart from the oligosaccharide group, prebiotics come from the crude fiber group. Inulin is one of the most commonly used fiber groups as a prebiotic [7].

The addition of Inulin as a prebiotic can increase the probiotic population and maintain it in a longer static phase [8], maintain the viability of the probiotic during storage, be well accepted by sensory organs, and can pass through the gastro-intestinal tract, reduce the risk of carcinogenesis and improve the management of inflammatory bowel disease [9]. Inulin has been reported to improve Rainbow trout's growth and biochemical parameters [10]. Prebiotics and probiotics can be applied separately or simultaneously (synbiotics). The presentation can also be found in liquid, powder, or capsule preparations, but probiotic preparations in liquid form are very easily contaminated and damaged due to environmental stress [11], [12]. Probiotic preparations in microcapsules are expected to protect probiotics from environmental stress and facilitate application [13], [14].

Some of the methods used in forming synbiotic microcapsules are spray drying, the emulsion method, and the extrusion method [15]. The disadvantage of the spray drying method is that the decline in the probiotic population is very high due to high-temperature stress in this process, but this method can be used on an industrial scale. While the emulsion and extrusion method does not use high temperatures, it is even very safe to protect the enzymes produced by probiotics. The shelf life of probiotics from the emulsion method is shorter than the extrusion method [16], although commercial use is still under study. The extrusion method generally uses alginate as a probiotic wrapper [17]. Alginate can be obtained from almost all types of seaweed, including Laminaran and Sargassum. Apart from being a source of alginate, Sargassum also has many benefits for the organism, such as antioxidant, antibacterial, and antiviral [18], [19]. Probiotic viability is a great concern in every probiotic preparation; equally important are the size and characteristics of the synbiotic microcapsule preparation [20].

Therefore, the purpose of this study was to determine the best concentration of Inulin for the growth of *Bacillus subtilis* BR610 isolated from the intestines of Rabbitfish, analyze the size and characteristics and viability of synbiotic microcapsules using alginate from Sargassum seaweed as a coating and inulin as a prebiotic on temperature and bile fluid stress.

II. Materials and Methods

A. Materials

Bacillus subtilis BR610 used in this study has undergone the selection stage of probiotic candidates from many isolates isolated from the intestines of Rabbitfish, *Siganus guttatus*. The alginate used in this study was extracted from *Sargassum* sp using the alginate extraction method through the calcium alginate pathway [21] Inulin is produced By Now Sports - Nutrition and Wellness, fructooligosaccharide extracted from blue agave. Bile is obtained from the bile fluid of Rabbitfish reared in floating nets. Before use, this bile is stored in a freezer -20ºC. Media and chemicals from Nutrient broth (NB) and Tryptic Soy Agar (TSA) from Difco; NaCl, $CaCl_2$, and $NaHCO_3$ from Merck. All media used are first sterilized using an autoclave with temperature conditions of 121ºC and pressure of 1 atm for 15 minutes.

B. Probiotic Growth Patterns with Additions Inulin

Growth patterns of *Bacillus subtilis* BR610 probiotic isolates isolated from the intestines of selected Rabbitfish obtained from the first activity were observed [8]. Isolates of *B. subtilis* BR610 were first rejuvenated on NB media by incubating for 24 hours on a swaying incubator. After that, probiotic cells were harvested by centrifuge at 4500 rpm for 15 minutes at 4ºC. The formed pellets are washed using saline solution two times. This formed pellet will be used to see its growth pattern on NB media by adding inulin according to treatment.

The concentrations of inulin used at this stage of the study were 0.05%, 0.1%, and 1% [13]. NB media without the addition of inulin is used as a control. Bacterial isolates that had been centrifuged were previously inserted into Erlenmeyer containing 10 mL of NB media, according to treatment, of 100 µL each. Next, the bacteria are incubated at 37ºC on a swaying incubator water bath. The bacterial population (total plate count / TPC) is observed every first 4 hours to 12 hours, then continued every 24 hours. The medium used for TPC observation is the Tryptic soy agar (TSA) plate. The best inulin concentration obtained from this study will be used at the microencapsulation stage of *B. subtilis BR610*.

C. Microencapsulations of Probiotic Bacillus subtilis BR610

Microencapsulation of probiotic BR610 was carried out using the destruction method. The treatment tested at this stage is the concentration of alginate used to coat probiotics: A = 1%, B = 2%, C= 3%. The probiotic microencapsulation procedure uses the [22] method without chitosan coatings. *B. subtilis* BR610 isolates were each cultured in 100mL NB media and then incubated on a swaying incubator for 24 hours. After 24 hours, the cells were harvested by centrifugation at 4500 rpm for 15 minutes at 4ºC. The pellets obtained were rinsed twice with a 0.85% sterile physiological solution. The cells were resuspended using a 5 mL physiological solution.

Encapsulation is done by adding 20 mL of 1% inulin solution (according to the best results from previous tests) and then incubating for 10 minutes in a swaying incubator. Furthermore, this inulin probiotic solution is put into 75 mL of alginate solution prepared according to the treatment. The amount of Inulin and alginate added is calculated according to the final volume of the suspension, which is 100 mL. This mixture of probiotic cell suspension, Inulin, and sodium alginate was then homogenized on a stirrer plate for 20 minutes. After homogeneous, each of these synbiotic mixtures was sampled for density by taking 1 mL and then diluted in stages using a physiological solution (0.85% NaCl). The probiotic population in this alginate mixture was observed by embedding samples onto a TSA media plate.

Furthermore, this synbiotic mixture was printed using a syringe volume of 10 mL with a needle size of 24G. The droplets of this synbiotic mixture are dropped into a 0.45 M $CaCl_2$ solution and gently stirred on a hot plate stirrer. This is done until the synbiotic mixture is wholly molded. The obtained synbiotic microcapsule beads are separated using Whatman filter paper with filtration and washed twice with distilled water. The beads of these synbiotic microcapsules are weighed and stored at 4ºC.

D. Observation of Morphology and Size of Microcapsules Synbiotic

The morphology and size of the synbiotic microcapsules obtained were carried out [23]. One hundred seeds from the microcapsules obtained were observed under a BX41 Light microscope equipped with a camera and grain diameter using the DP21-SAL application. Observation of synbiotic microcapsule viability, release speed, encapsulation rate, and absorption efficiency of synbiotic microcapsules.

To determine the trapped bacterial population, the population of synbiotic stock bacteria is first observed to be encapsulated. Observation of probiotic populations began during the rejuvenation process in nutrient broth, then the density of probiotics after mixing with prebiotic and alginate (N_0) and printing into synbiotic microcapsule beads (N_t).

Bacterial populations were observed using the total plate count method of the TSA plate media. Bacterial colonies grown after 24-48 hours of incubation were counted manually and then tabulated using the equation of [24]:

$$\text{CFU/mL} = \frac{\text{Average growing colony}}{\text{volume spread into Petri dishes}} \text{ x } \frac{1}{\text{dilution factor}} \quad (1)$$

Observation of probiotic viability in synbiotic microcapsule beads and probiotic release rate from synbiotic microcapsules refers to the method [25]. Release velocity is the time synbiotic microcapsule beads in an alginate crushing solution dissolve completely. The absence of synbiotic microcapsule beads and discolorations of the alginate-destroying liquid characterizes it. A total of 1 gram of synbiotic microcapsule beads (each of each alginate concentration) is put into the alginate granule crushing solution and shaker for 10, 20, and 30 minutes. After incubation, dilution is carried out in stages using physiological solutions. Then bacterial enumeration (N_t) is carried out by taking 100 µL of dilution (10^{-4}, 10^{-5}, and 10^{-6}) spread on TSA plate media. After that, it was incubated for 48 hours at a temperature of 37ºC. Inoculation of samples is carried out duplo. The plate count method calculated the total colony, and the bacterial density of each 1-gram microcapsule formed (N_t) was calculated using the equation [24]. The highest TPC data obtained on this release velocity observation were then fed into an equation [24] to determine the probiotic encapsulation rate:

$$\text{EY} = \left(\frac{Nt}{N0}\right) \text{ x } 100 \quad (2)$$

Where:
Nt = number of trapped bacteria/number of bacteria in the synbiotic microcapsule (CFU/mL).
N0= number of bacteria to be encapsulated (CFU/mL).

The bacterial absorption/encapsulation efficiency of the alginate matrix can also be estimated by taking the highest population data of each alginate concentration on the observed release velocity and tabulated into an equation [26]:

$$\text{Efficiency encapsulation (\%)} = \frac{P \text{ x } Q}{R} \quad (3)$$

P = probiotic population per gram of microcapsules (CFU/gram microcapsules)
Q = mass of microcapsules resulting from the total synbiotic suspension used (grams)
R = total probiotics in cell-biopolymer suspension (CFU/mL)

E. Viability Test of Synbiotic Microcapsules against Bile Fluid Simulation and Temperature

A probiotic resistance test in synbiotic microcapsules was conducted [27]. Bile simulation (SGJ), the physiological solution was added with 10% bile and incubated for 60 minutes at 37°C. Bacterial viability is carried out by harvesting bacteria by centrifuge at 2000 rpm for 5 minutes. The precipitate formed is rinsed with ten sterile physiological solutions, then centrifuged again, and the precipitate is added with an alginate granule crusher solution. After the beads dissolve, this synbiotic microcapsule liquid is diluted in stages and then implanted into TSA plate media. The samples were then incubated at room temperature for 48 hours. The equation manually calculates the number of bacteria grown [24].

Independent synbiotic and probiotic microcapsules were tested for heat resistance at 70°C for 60 minutes and 90°C for 30 minutes (simulated conditions in a feed molding machine). Synbiotic microcapsules of 0.1 g and 0.1 mL of free cell suspension were transferred to test tubes and incubated in a water bath according to treatment. After heat treatment, the test tube is cooled to room temperature (25°C). The survival of encapsulated probiotics and free cells was observed by implanting them into a media plate as in the stages of the previously described method [28]. Survival ratios were calculated as the log-transformed ratio between viable counts per vial before and after the simulated salt bile and temperature (log N/N0).

F. Data analysis

The morphological form of the synbiotic microcapsule beads obtained is displayed as photos/pictures. The quantitative data obtained were analyzed using a one-way analysis of variance and significance of the difference between the mean determined by the Tukey multiple range test ($P<0.05$) using SPSS. Results are presented as standard deviations (SD) from replicated determinations.

III. Results and Discussion

A. Results

1) Probiotic growth pattern with the addition of inulin: Growth patterns of BR610 probiotic isolate at several inulin concentrations can be seen in Table 1.

TABLE I
THE PROBIOTIC POPULATION OF B. SUBTILIS BR610 WAS ISOLATED FROM THE INTESTINES OF RABBITFISH, SIGANUS GUTTATUS, IN NUTRIENT BROTH MEDIA WITH INULIN ADDITION TREATMENT

Incubation (hours)	Population in Inulin (%)				Average
	0	0,05	0,1	1	
4	8.489	7.787	8.169	7.743	8.047±0.35[a]
8	9.520	9.416	9.168	8.975	9.269±0.25[c]
12	9.309	8.813	8.666	8.673	8.865±0.30[c]
24	8.958	9.041	8.960	9.210	9.042±0.12[c]
48	8.933	8.891	9.316	9.389	9.132±0.26[c]
72	8.728	8.814	9.433	9.494	9.117±0.40[c]
96	8.561	8.893	8.721	9.149	8.831±0.25[bc]
120	7.398	8.000	8.343	8.857	8.149±0.61[ab]
144	7.547	7.371	7.643	8.373	7.734±0.44[a]
168	7.472	7.031	7.806	8.073	7.596±0.45[a]
Average	8.491±0.77 [ab]	8.405±0.80[a]	8.622±0.62[ab]	8.793±0.58[b]	

* Values with the same superscript letter in the same row/coloum are not significantly different ($p>0.05$).

It was seen that the population of B. subtilis BR610 began to increase to 109 CFU/mL after incubating for 8 hours and lasted up to 72 hours. The probiotic population began to decline with 98 hours of incubation (108 CFU/mL). In subsequent observations (120 hours), the probiotic population in the 1% inulin addition treatment tended to be 8.86±0.7 log CFU/mL higher than other treatments. The statistical analysis showed that adding 1% inulin significantly affected the BR610 probiotic population, with the highest incubation period from 8 hours to 72 hours, although no interaction was found between inulin concentration and incubation period. Based on the data in this table, it was decided to take a 1% concentration of inulin to be used later, namely when printing synbiotic microcapsule beads.

2) Morphology and size of synbiotic microcapsules: Morphological visualization of BR610 synbiotic microcapsule beads printed using a 10 mL volume syringe with a 24G needle can be seen in Figure 1. Generally, the synbiotic microcapsule beads produced are oval, round, and clear white, with elasticity/viscosity increasing with the amount of alginate given.

Fig. 1 Visualization of BR610 synbiotic microcapsule beads using 5x and 10x magnification light microscopes. From left to right, alginate 1%, 2%, and 3%.

Based on Figure 2, the diameter of the synbiotic beads increases with the amount of alginate administered. Adding 3% alginate gives a significantly higher grain diameter than 1% but is not significantly different from adding 2% alginate.

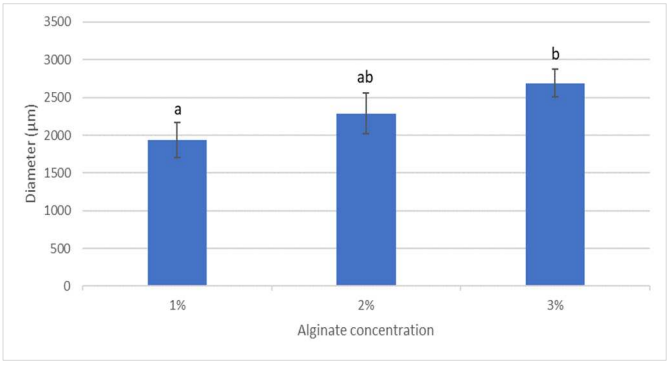

Fig. 2 Beads diameter of synbiotic microcapsule isolate of *Bacillus subtilis* BR610 isolated from the intestines of Rabbitfish, *S. guttatus*.

3) Viability, release speed, encapsulation rate, and absorption efficiency of synbiotic microcapsules: The viability of probiotic BR610 after encapsulation in alginate dressing ranges from 10^7-10^8 CFU/mL. The release rate of the probiotic at the time of crushing in an alginate granule crushing solution [25] is approximately 15->20 minutes. The release speed of alginate beads is directly proportional to the alginate concentration. The rate of probiotic encapsulation in the 2% alginate treatment was significantly higher than the 1% treatment, and the 1% alginate treatment was higher than the 3% treatment. Similarly, with encapsulation efficiency, the 2% alginate treatment is more efficient than the 1% and 3% treatments (Table 2).

TABLE II
PROBIOTIC VIABILITY, ENCAPSULATION RATE, AND ABSORPTION EFFICIENCY OF B. SUBTILIS BR610 PROBIOTIC ISOLATED FROM THE GUT OF RABBITFISH, SIGANUS GUTTATUS, IN SYNBIOTIC MICROCAPSULE BEADS

Variable	Alginate concentration (%)		
	1	2	3
Volume suspense inulin 1% + alginate (mL)	50	50	50
Cell population (colony/mL cell suspension)	7,873±0,56	8,164±0,08	8,427±0,2
Total number of cells in suspension (colony)	9,572±0,56	9,863±0,08	10,125±0,2
Mass of beads produced (grams)	30,2±2,09	39,5±3,1	45,0±3,95
Beads diameter (mm)	1,9±0,2a	2,3±0,2ab	2,7±0,2b
Release speed in bead crushing solution (minutes)	15	20	>20
Viability of probiotics in beads (log CFU/gram)	7,286±0,71a	7,776±0,06a	7,703±0,26a
Encapsulation rate (%)	26,8±7,9b	40,9±1,9c	19,1±3,3a
Encapsulation efficiency (%)	16,3±5,4a	32,3±1,2b	17,2±3,5a

4) Viability of synbiotic microcapsules in bile fluid simulation and temperature: The viability of probiotics in BR610 synbiotic microcapsule beads after incubating for 90 minutes in a physiological solution and supplemented with 10% Rabbitfish bile can be seen in Figure 3. The viability of probiotics in alginate beads was 2% higher (7.9 x 10^6 CFU/ml), significantly higher than the 3% (2.2 x 10^5 CFU/ml) and 1% (5.3 x 10^3 CFU/ml) treatments.

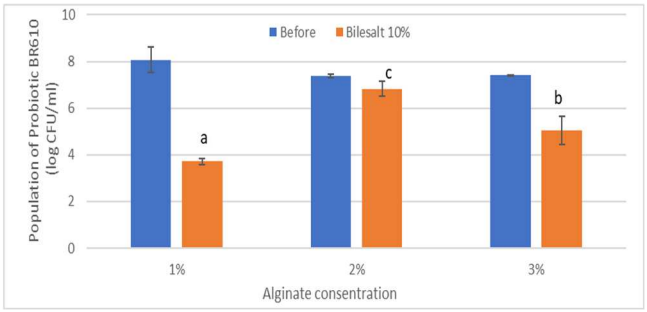

Fig. 3 Viability of probiotic *B. subtilis* BR610 in alginate beads after incubation at simulated 10% bile fluid for 90 min

The temperature stress treatment in this study refers to the condition of the fish feed in the molding machine that will be used later. These fish provide a moulding machine that has increased temperature in the feed material reservoir and at the end of the feed moulding tool where the pellets are formed. The average temperature in the tube of this fish pellet machine is around 70-90°C. Based on this information, the trial at 70°C was incubated for 60 minutes, while the 90°C temperature treatment was incubated for 30 minutes. Based on the data obtained in Figure 4, the viability of probiotics in synbiotic microcapsule beads is highest in beads with an alginate concentration of 2% after incubation at 70°C. In contrast, in those incubated at 90°C, the highest is obtained in beads with an alginate concentration of 3%. The statistical analysis results show that probiotics' survival in alginate beads is significantly higher than probiotics without alginate/free cell coated.

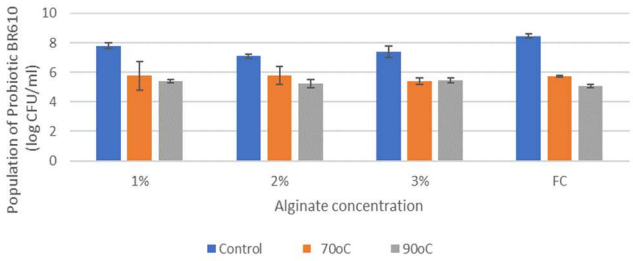

Fig. 4 Viability of probiotic *B. subtilis* BR610 in alginate beads after incubation at 70°C and 90°C.

B. Discussion

Inulin has been widely applied as a prebiotic derived from one of the fibre groups. Adding inulin to the nutrient broth media can increase the population of *Bacillus subtilis* BR610 in this study and higher than reported by [7]. However, the results of the study also found that the treatment of adding inulin to the nutrient broth media was able to increase the population of *B. velezensis* compared to the addition of glucose, raffinose, and lactulose. The addition of Inulin as a prebiotic source to observe the growth of *Lactobacillus casei, Lactobacillus plantarum*, and *Lactobacillus rhamnosus* was also reported to be better than the addition of oligofructose and potato starch [26]. In addition to increasing bacterial populations, the addition of inulin can also increase the antibacterial activity of *Lactobacillus spp., Bacillus subtilis*, and *B. valezensis* [29], as well as extend the shelf life of probiotics [30]. This increase in antibacterial activity is because inulin can maintain the static phase of the probiotic [8]. The static phase of growth of *B. subtilis* BR610 in this study was achieved after an incubation period of 24-96 hours. Even at 1% inulin treatment, the probiotic population remained high for up to 168 hours.

Based on the results of observing the growth pattern of probiotic BR610 in this NB + inulin media, then in the microencapsulation process, the concentration of inulin used is 1%. Synbiotic microencapsulation using the extrusion method has been reported by [22], [26]. The granular shape of the alginate microcapsules found in this study has similarities to those previously reported by researchers, namely round, transparent white [31], [32]. Alginate beads' structural and mechanical properties are determined mainly by the concentration of alginate or calcium chloride [33], [34]. This structural property can be seen in the difference in shape and suppleness of the grains we produce in this study. At 1% alginate concentration, its structural properties are more-flabby/wrinkle-looking than concentrations of 2% and 3%. This structural property is influenced by the lower viscosity of 1% alginate.

The diameter of the microcapsule beads obtained in this study is quite large because the beads measured are BR610 synbiotic microcapsule beads that are still wet. Similar grain diameters were also reported by [35], i.e., 1.86 – 2.25 mm, for 1-2% alginate beads added with inulin. The concentration of inulin added to the alginate solution also affects the diameter of the beads, where the addition of 1% inulin to the 2% alginate solution produces 2 mm microcapsule beads that are significantly lower than the addition of 2% inulin [35], as well as the size of the diameter of the microcapsule produced when making oral vaccines for fish using the extrusion method is 3.5 mm [36]. The size of the microcapsule grain diameter using the extrusion method is strongly influenced by factors such as alginate concentration, calcium chloride concentration, needle diameter, pump pressure, the distance between the needle and calcium chloride solution, droplet speed, stirring speed, water-oil ratio [24]. The diameter of these microcapsule beads will also decrease when the beads are dried [37]. The diameter of microcapsule beads with an alginate concentration of 2% when wet is 2 mm, and after drying using hot wind, it becomes 0.9 mm, while those using a freeze dryer become 1.34 mm. The results of this study showed that the diameter and chewiness of the beads increased along with the concentration of Na-alginate used.

The encapsulation rate obtained in this study ranged from 19-40%, similar to that reported by [38], [39]. The best encapsulation rate and efficiency were obtained at intermediate concentrations (2% alginate + 1% inulin), with the viability of probiotic BR610 ranging from 7.776±0.06 log CFU/mL. Similar research results were also delivered by Olivares and Silva [20] who concluded that the best encapsulation efficiency can be obtained at medium viscosity. According to Dehkordi et al., [40], the extrusion method is the easiest probiotic encapsulation method and can reduce cell damage, thereby increasing encapsulation efficiency. The extrusion method can also allow the incorporation of a large number of probiotic cells into a certain amount of encapsulation material, which can certainly increase the encapsulation efficiency [41].

After the synbiotic beads BR610 were exposed to 10% bile for 90 minutes, there was a decrease in the probiotic *B. subtilis* BR610 population. The decline in the population of probiotics

in beads may be due to bile entering through diffusion into alginate beads or porosity usually found in alginate beads. However, adding prebiotics can mask the porosity of alginate beads [42]. This allows the continuity of probiotic BR610 in the treatment of 2% alginate plus 1% inulin significantly higher (92.3±4.2%) than the treatment of 3% (68.0±8.1%) and 1% alginate (45.9±1.6). Another possibility that causes survival in the 2% alginate treatment is due to the ability of *B. subtilis* BR610 to form aggregates in beads due to the availability of suitable substrates [13].

The microencapsulation method can maintain bacterial cells after exposure to high temperatures compared to free cells without microencapsulation [43]. However, temperature can also affect the viability of probiotics (*Bacillus sp*) in synbiotic microcapsules so that they decrease. This is due to conditions not synchronized with the life tolerance of the *Bacillus sp* strain, which is optimal at temperatures around 33-55^{o}C and can tolerate temperatures up to 66^{o}C and will form spores but will experience a decrease in population [44]. Based on the results of this study, coating probiotic *Bacillus subtilis* using alginate can maintain the viability of probiotics so that when microcapsule beads get high-temperature stress, probiotics can still survive. This can be a reference for the application of probiotics in various aspects, such as in the manufacture of fish feed, where it is known that in the process of molding fish feed pellets, the temperature of the feed molding machine can reach temperatures of 70^{o}C or more, so this is no longer a barrier factor because probiotics have been coated with alginate.

IV. Conclusion

Inulin can increase the population of the probiotic *Bacillus subtilis* BR610 isolated from the intestines of Rabbitfish. The performance of microcapsule beads using 2% alginate and 1% inulin is better than free cells, 1%, or even 3% because these beads have a diameter that is still included in the category of microcapsule synbiotic beads, higher encapsulation rate, efficiency encapsulation, and can protect probiotics from bile salt and high temperatures.

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Conflict of Interest

We declare that they have no known conflict of interest or personal relationships that could have influenced the work reported in this paper.

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