Microencapsulation of Probiotic *Lactobacillus casei* based on Alginate and Chitosan Materials

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ABSTRACT--- *Probiotics are microorganisms that live in the stomach related plot which can give health advantages to the body. Probiotics must be able to survive in an acidic environment (pH = 2) during transit in the gastric to arrive the large intestine in adequate amounts (10^6 - 10^7 CFU/g) to allow for colonization and reproducing. However, most of these probiotics cannot survive in adequate amountsin acidic conditions. One way to protect probiotic cells under these conditions is through a microencapsulation system. Microencapsulation is done to protect probiotic cells from low pH, bile salts, etc. Lactobacillus casei includes probiotics that are very sensitive to pH 2-2.5. The non-encapsulated Survivability of L. casei is only 40.14% in liquid pH 2 from 8.75log CFU/g to 3.53log CFU/g while L. casei carried out microencapsulation can achieve survivability of 63.47% to 95.3%. Alginate is a microencapsulation material that is inexpensive and sensitive to changes in pH so that it is suitable as a microencapsulation of probiotics. The surface of porous alginate needs other ingredients that can be requested by the pores. Alginate is resistant to pH 2-2.5 and expands at neutral pH to alkaline, which results in increased alginate pores. Chitosan can be used as an alginate mixture in probiotic microencapsulation materials. Chitosan can bind by crosslinking with alginate, which is between the NH_2 group of chitosan and COO^- group of alginate.*

Keywords--- *Lactobacillus casei*; alginate; chitosan

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1. INTRODUCTION

Probiotics are microorganisms that live in the digestive tract that can provide health benefits for the body [1]. Probiotics must survive in an acidic atmosphere (pH = 2) while transiting the gastric so that they can reach the large intestine in sufficient quantities (10^6 - 10^7 CFU/g) to allow for colonization and breeding [2]. Therefore, usually, probiotics contained in functional foods/drinks must be in the range of 10^8-10^9 CFU/g right before consumption [3 -7]. However, most of these probiotics cannot survive in sufficient quantities in acidic conditions and/or are exposed to oxygen.

One way to protect probiotic cells in these conditions is through a microencapsulation system. Microencapsulation is the process of inserting probiotic cells into the matrix that can protect cells due to degradation and release them under control under certain conditions [8]. Also, microencapsulation is carried out to protect probiotic cells from low pH, bile salts, etc. during transit in the gastrointestinal system [9]. Much research has been done on microencapsulation of various types of probiotics such as *B. longum, B. animalis, L. plantarum, L. rhamnosus, L. acidophilus, L. gasseri, B. bifidum, B. breve, Saccharomyces boulardii, L. paracasei, B. lactis, L. bulgaricus, E. faecalis, B. adolescentis, L. casei* [10-21].

One type of probiotics commonly used in curd (yogurt) is *L. casei*. *L. casei* is a gram-positive, anaerobic bacterium, has no locomotor, does not produce spores, is rod-shaped, and is one of the bacteria that play an important role in digestion. *L. casei* is a bacterium that can break down proteins, carbohydrates, and fats in food and helps the absorption of important elements and nutrients such as minerals, amino acids, and vitamins that humans and animals need to survive [22].

L. casei bacteria are 0.7 - 1.1 x 2 - 4 µm and are important bacteria in the formation of lactic acid. Like other lactic acid bacteria, *L. casei* is acid-tolerant, cannot synthesize perforin, and ferment with lactic acid as the main final metabolite. These bacteria form clusters and are part of a facultative heterofermentative species. Growth of *L. casei* at 15°C and requires riboflavin, folic acid, calcium, pantothenic acid, and other growth factors. *L. casei* is an adaptable species, can be isolated from fresh and fermented livestock products, fresh and fermented food products. *L. casei* has a role as a probiotic in humans, an acid-producing starter culture for milk fermentation, a special culture for intensification, and acceleration of flavor development in varieties of cheese affixed with bacteria [23, 24].

L. casei is found in fermented milk and has beneficial health properties for humans. *L. casei* can reduce diarrhea and help modify microflora in the body. *L. casei* produces DL-lactic acid and amylase which complements the growth of *Lactobacillus acidophilus*. Most *L. casei* strains can ferment galactose, glucose, fructose, mannose, mannitol, N-

acetylglucosamine, and tagatose. The ability to ferment lactose is less common in strains isolated from vegetable matter than those from cheese and the human digestive tract [23].

L. casei is thought to be able to control organisms that can cause toxic effects in the human digestive tract, including *Escherichia coli. L. casei* can inhibit the growth of *H. pylori* and help microflora in the large intestine [25].

Microcapsules as microencapsulated products consist of semipermeable or nonpermeable, round, thin, and strong membranes that surround solid or liquid nuclei with very small and varied diameters, which are several microns to 1 mm. Examples of microencapsulation can be seen in Figure 1 below.
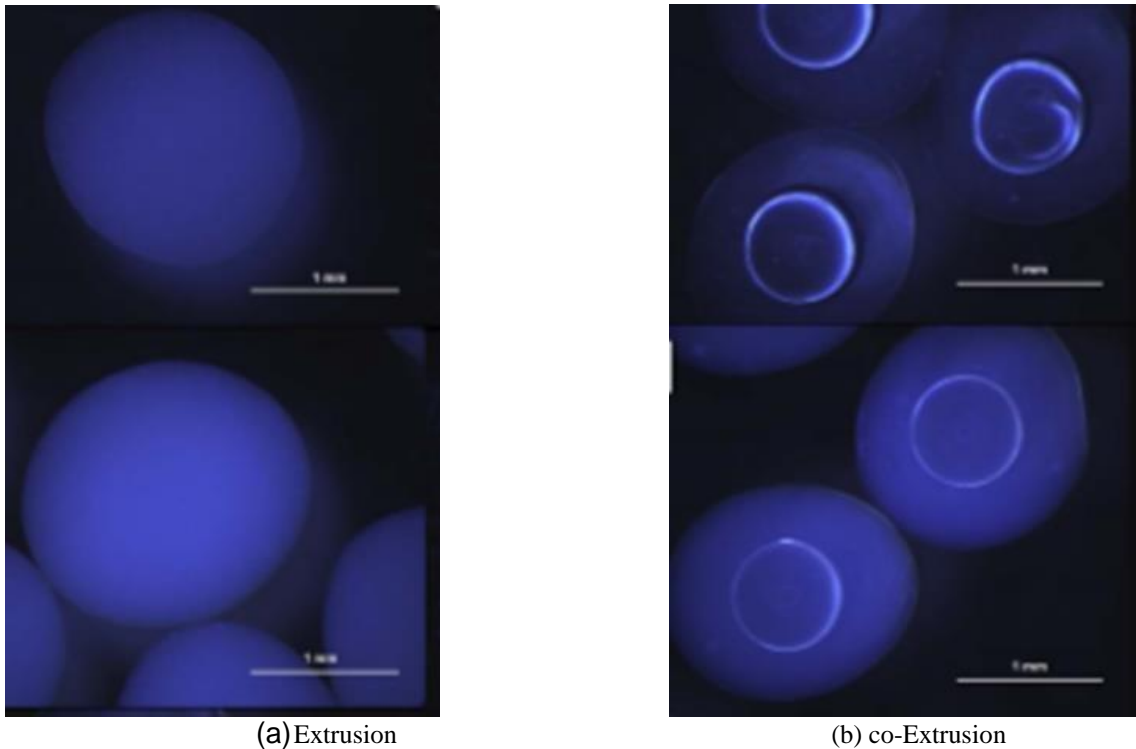

(a) Extrusion   (b) co-Extrusion

Figure 1. Probiotic encapsulation with extrusion (a) and co-extrusion (b) methods [21]

Microcapsule material is a safe material used in food applications such as carboxymethyl cellulose, xanthan gum, starch, carrageenan, gelatin, pectin, casein, whey protein, alginate, and chitosan [26-30].

Several techniques have been applied to increase the resistance of microorganisms that are sensitive to gastric acid/SGF (simulated gastric fluid) so that the probiotic microencapsulation is successful. Some of these are considering the nature of probiotic strains, resistance to acids, their encapsulation materials and concentrations [19], the incorporation of several food-grade polymers into the alginate matrix, and microencapsulation techniques[9, 31-37].

The encapsulation system is believed to be able to protect L. casei in extreme environments so that it can work effectively until it reaches the intestine in maintaining digestive health as shown. The condition of the human digestive system is shown in Figure 2 below**.**
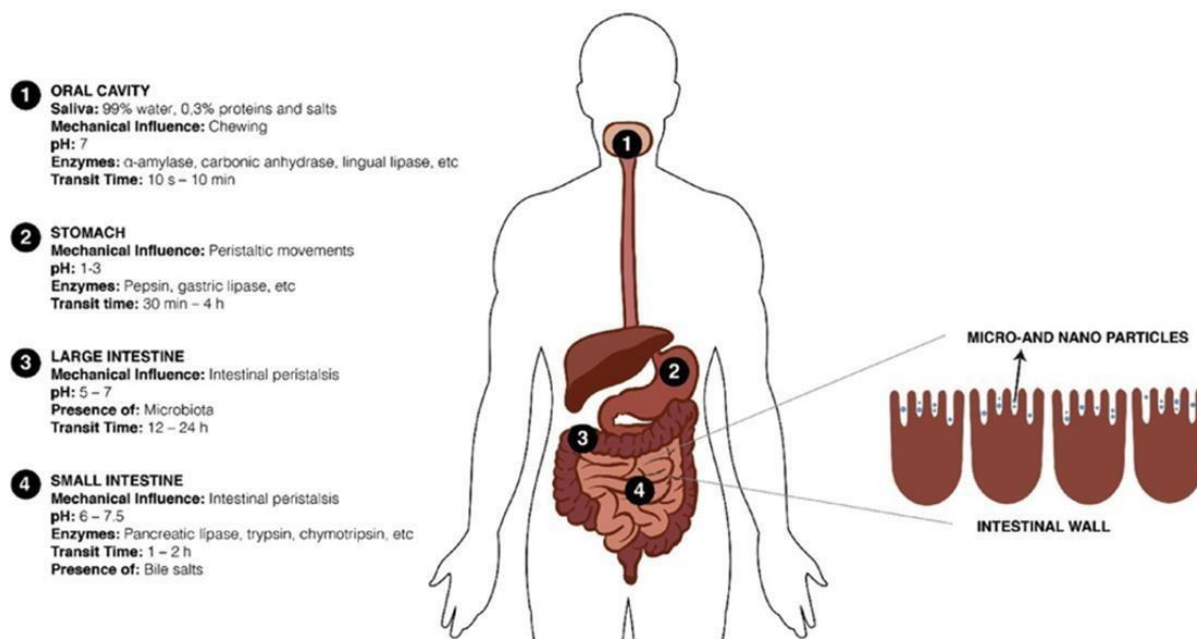
Figure 2. Diagrammatic representation of the main physiological conditions in the human digestive system [36]

2. MICROENCAPSULATION MATERIALS

There has been a lot of probiotic microencapsulation research using various food bases such as alginate and chitosan.

### 2.1 Alginate-Based Microencapsulation Materials

It has been evaluated to immobilize probiotics, alginates are the most commonly used matrix, this is due to biocompatibility and economical efficiency [35]. Some of the advantages of alginate particles are porosity and high durability in an acidic environment [38]. However, alginate porosity can be a weakness in its application as a probiotic drink. The presence of pores provides a way for gastric acid to enter the microcapsules so that there is a great chance of killing probiotics encapsulated by alginate. Therefore, the combination of alginate with polymers or other foodstuffs is needed to overcome the porosity [39, 40].

Alginate is a natural polymer that has been successfully applied as a pH-sensitive material for microencapsulation of probiotic bacteria [39]. Alginate is a polysaccharide extracted from algae consisting of various amounts and sequential distribution of β-D-mannuronic (M) and α-L-guluronic acid (G) (copolymers containing MM, GG, and irregular sequences of M and G units) which can influence the functional properties of alginate as a supporting material [40]. When a sodium alginate solution containing cell suspensions is poured into a calcium solution, bound ions interact with other GG blocks to form complexes that lead to gel formation and possibly release of cells that are in the intestinal tract [41]. Alginate microencapsulation strategies have been established to protect the viability of probiotics in gastrointestinal digestion [42]. Figure 3 below shows alginate and its chemical structure.

Table 1. The effects of alginate-based microencapsulation on the protection and viability of *L. casei* probiotics in SGF at pH 2, incubation time 120 min, and temperature 37°C

Microencapsulation technique	Microencapsulation materials type	Survivability of Probiotic (%)	Reference
Extrusion	Fee cell	40,14	[10]
	Pea isolate-alginate (Fresh capsule)	95,3	
	Pea isolate-alginate (Preserved capsule)	74,31	

L. casei = Lactobacillus casei
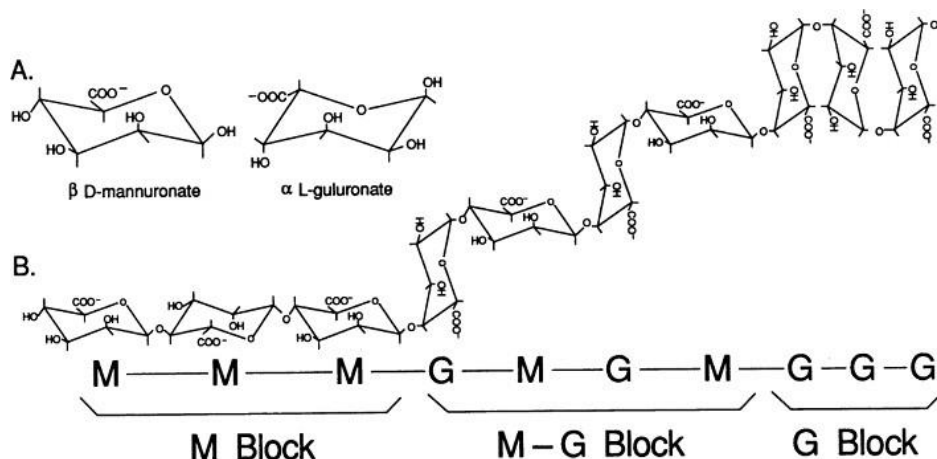
(a) Three-dimensional structure of alginate [43]
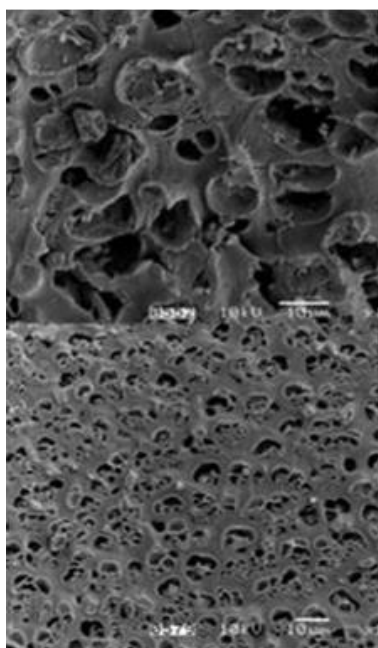
(b) Alginate Photos [21]

Figure 3. Three-dimensional structure of alginate (a) and alginate photo (b)

The results of the study [10] showed that *L. casei* free cells experienced a significant decrease in viability (5.22 log CFU/g) after incubation in SGF for 2 hours. This is by other studies that showed that *L. casei* ATCC 393 strains were sensitive to acids [5, 44]. The amount of viability of *L. casei* in fresh capsules did not show a significant decrease after incubation in SGF for 2 hours, which is only 0.41 log CFU/g. While the viability of L. casei in preserved capsules decreased significantly by 2.24 log CFU/g after incubating in SGF for the first 60 min, then the stable cell count was 0.03 log CFU/g for 60 to 120 min. These results indicated that *L. casei* in preserved capsules were more sensitive to SGF than fresh capsules.

2.2 Material of Chitosan-based Microencapsulation

Processing of chitin with an alkaline solution turns it into either fully or partially deacetylated chitosan. Chitosan can be defined as a natural, non-toxic biopolymer and linear polysaccharide consisting of β-1,4-GlcNAc and β-1,4-GlcN. Chitosan is insoluble in water but soluble in aqueous organic acid solutions [45]. Unlike chitin, chitosan is not a component in animal species and is rarely found in nature. Natural sources of chitin, including crab and shrimp shells, squid, bone plates, and cuttlefish do not contain chitosan, however, fungi synthesize chitin and chitosan on their cell walls [44]. Chitosan is an important component of *Zygomycetes* cell walls [46]. Chitosan is also naturally found in

mycelia, stems, and spores of *Basidiomycetes, Ascomycetes*, and *Phycomycetes*[47]. Chitosan from *crustacean* sources has a high molecular weight (MW) with a low polydispersity degree of N-acetylation (DA) below 20% and a 1% solution viscosity from 500-1,700 cps. While chitosan mushrooms have low MW with high polydispersity, DA is lower than 15% and the viscosity of the solution is 1% of 10-15 cps [44]. Every year, around 150,000 tons of chitosan that can be used industrially comes from the conversion of chitin obtained as a by-product of seafood processing. Most chitosan is used in cosmetics, organic fertilizers, and food supplements [48]. Chitin and chitosan can be distinguished based on the number of D-glucosamine acetylation units. Chitin contains more than 70% acetate units, while chitosan has less than 30% acetylation. With organic acids such as formic acid, acetic acid, and ascorbic acid, chitosan forms a salt and consequently becomes soluble in water [45]. Chitosan contains three reactive functional groups, an amino group or N-acetamide, together with two primary and secondary hydroxyl groups at positions C-2, C-3, and C-6 respectively. The main difference between the structure and physicochemical properties of the different chitosan is the amino group or N-acetamide [45]. Chitosan can be classified according to N-acetate (FA), DA, polymerization (DP), MW, MW (PD or Polydispersity), residue fraction, and N-acetylation pattern (PA) or sequence. Chitosan offers great potential for applications in various industries due to typical physicochemical characteristics such as biocompatibility, biodegradability, and low toxicity [45]. Figure 4 below shows the chemical structure of chitosan.
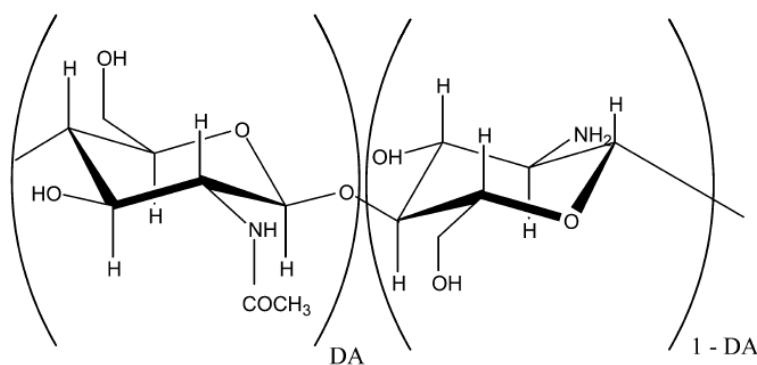

Figure 4. Structure of chitosan with acetylation degree (DA)[49]

Chitosan can form a gel with polyphosphate or sodium alginate (multivalent nontoxic anion contraction) by ionic crosslinking [50]. The alginate microcapsule layer and its efficiency in protecting probiotics have been widely studied for several years. Previous studies have found that the alginate microcapsule layer with chitosan has a significant effect on alginate stability, thereby increasing the viability level of encapsulated probiotics[35].

Table 2. The effects of chitosan-based microencapsulation materials on the protection and viability of *L. casei* probiotics in SGF at pH 2, incubation time of 120 min and temperature of 37°C

Microencapsulation Technique	Microencapsulation Materials Type	Survivability of Probiotic (%)	Reference
Extrusion	Alginate	84,34	[5]
	Alginate-Chitosan	87,54	
	Alginate-Chitosan-CMCS	93,83	
	Free cell	49,83	[14]
	Alginate	63,47	
	Alginate-Oligofruktosa DP 2-10	68,19	
	Alginate-Inulin DP 12	69,04	
	Alginate-Inulin DP≥23	69,79	
	Alginate-Chitosan	78,45	
	Alginate-Oligofruktosa DP 2-10- chitosan	84,05	
	Alginate-Inulin DP 12-chitosan	84,66	
	Alginate-Inulin DP≥ 23-chitosan	85,07	

L. casei = Lactobacillus casei
CMCS = *carboxymethyl chitosan*
DP = Degree of polymerization

That has been encapsulated from *L. casei* probiotics with extrusion techniques and tested on microcapsules in SGF at 37°C [5, 44]. The results of the study [5] showed that at pH 6.5 (control), the viability of *L. casei* cells in SGF

remained above 8log CFU/g after 2 hours of incubation at 37ºC whether encapsulated or not. At pH 2, no free cells survived after 2 hours incubation in SGF and the results show that *L. casei* was sensitive to an acidic environment (pH 2). Under the same conditions (pH 2, 30 min), the numbers of *L. casei* live cells in alginate microcapsules, alginate chitosan microcapsules and alginate chitosan-CMCS microcapsules were 7.97, 8.09, and 8.43log CFU/g and with an increase in time of up to 120 min, the decrease in the three types of microcapsules was 0.86log, 0.71log, and 0.52log, respectively. Viability of *L. casei* under acidic conditions showed that there was a decrease in bacterial-like CFU/g at pH 2 and 3. It was clear that the viability of encapsulated cells was significantly better than free cells after exposure to SGF (pH 2) and alginate microcapsules. Chitosan CMCS can effectively protect *L. casei* against an acidic environment. In the presence of a 0.5% bile solution, the survival rate of *L. casei* was encapsulated in alginate microcapsules, alginate-chitosan microcapsules, alginate chitosan-CMCS microcapsules are 67.4%, 87.9%, 91.1% after 6 hours of exposure while a free cell is 0.1%. After exposure to 1% bile solution for 6 hours, the survival rate of *L. casei* encapsulated in alginate-chitosan-CMCS microcapsules was 85.4% while the free cells were 0.03%. These results indicated that the resistance of various *L. casei* microcapsules to bile solutions was higher than free cells. The formation of hydrogels around cell pellets was considered the basis of cell protection. This was because acidic liquids and bile salts need to seep through the gel layer before reaching the cells [31]. The results showed that alginate-chitosan-CMCS microcapsules were the most effective in protecting probiotic bacteria from acidic liquids and bile salts and making this approach potentially useful for shipping probiotic cultures to the human digestive tract.

Coating with chitosan was able to provide the best protection for cells compared to the incorporation of inulin into alginate microcapsules (without chitosan coating)[14]. The addition of different lengths of inulin chains (mainly HP-inulin) together with the coating of chitosan, significantly affected the viability of probiotic bacteria during testing of gastrointestinal fluid and bile salts, when compared with alginate, alginate/inulin, alginate/chitosan, and free cells. The results of the study showed that the viability of microencapsulated cells in alginate and encapsulation microcapsules was significantly higher than free cells [14]. The initial cell count of *L. casei* was $6.8 \pm 2.3 \times 10^{13}$ CFU/mL, while after encapsulation, the cell count ranged from $2.4 \pm 0.5 \times 10^{12}$ and $5.7 \pm 0.6 \times 10^{12}$ CFU/g microcapsules. The number of probiotics decreased significantly during incubation time and the reduction rate was significantly greater in free cells (P <0.05). Reduction of about 3.7log in cell viability from *L. casei* co-encapsulation with different prebiotic chain lengths from inulin after exposure to in vitro acidic conditions (pH ~ 1.5) for 2 hours, compared with 4.5log reduction for Alginate encapsulation and 6.9log for free cells. A reduction of about 1.28log was seen in the viability of *L. casei* cells encapsulated with alginate/inulin/chitosan after exposure to acidic conditions in vitro, compared to cells encapsulated with alginate/chitosan (without prebiotics) reduced by 2.6log. Cells that are encapsulated with alginate and encapsulated together with all prebiotics are more resistant than free cells after sequential incubation in SGF and SIF. The results of the study showed that chitosan coating increased the resistance of encapsulated cells under sequential conditions. Reduction in cell viability of about 3.6log for alginate-coated chitosan after 120 min of sequential incubation and a reduction of about 2.7-2.9log in cells for chitosan-coated alginate/inulin compared to a 4.7-4.9log reduction for alginate/inulin (without chitosan coating), 5.7log reduction for alginate encapsulated and 6.7log for free cells [14]. As a result, the incorporation of various inulin prebiotics with different length chains, during probiotic microencapsulation increases the resistance of organisms at low pH, resulting in higher cell counts, when compared to microcapsules produced only with alginate. Although long-chain inulin, not significantly (p > 0.05) affected microencapsulation cell viability at low pH, HP (high polymerization) -inulin seemed to be slightly more effective than other long-chain inulin. This may be because long-chain fraction fermentation was slower and stable in the range of pH and ionic strength in the human digestive tract [49][51].

3.   CONCLUSIONS AND SUGESTIONS

3.1 Conclusions

Lactobacillus casei is a probiotic that is very sensitive to pH 2-2.5. Survivability of *L. casei* that was not encapsulated was only 40.14% in liquid pH 2 from 8.75log CFU/g to 3.53log CFU/g, so it was no longer effective to allow for colonization and propagation (the limit was 6log CFU/g). While *L. casei* performed microencapsulation can achieve 63.47% to 95.3% survivability, depending on the material formulation and microencapsulation technique.

Alginate is an inexpensive microencapsulation material that is sensitive to pH changes, making it suitable as a probiotic microencapsulation material. The surface of the alginate is porous, so it requires other materials that can cover the pores. Alginate is resistant to pH 2-2.5 and expands at a neutral to basic pH, which increases in alginate pores. Chitosan can be used as an alginate mixture in probiotic microencapsulation materials. Chitosan can bind by crosslinking with alginate, which is between the NH_2 group on chitosan and COO^- group on alginate. Meanwhile, chitosan cannot be used as a probiotic microencapsulation material singly.

3.2 Suggestions

Chitin nanofiber is a polymer that has biodegradable, nontoxic, and acid-resistant properties that can be considered to cover the shortage of acid-soluble chitosan as encapsulation material. Previous studies have not found the microencapsulation process of *L. casei* using the composite system of nanofiber Chitin and chitosan alginate systems. Therefore research is needed about this in the future.

4. ACKNOWLEDGMENTS

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