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USING *Sargassum* SEAWEEDS GROWN IN COASTAL AREAS OF SOUTH ASIA, AS COATING MATERIALS AND ASSESSING THE SURVIVABILITY OF MICROENCAPSULATED PROBIOTIC BACTERIA IN DAIRY BASED FOOD PRODUCTS

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ABSTRACT

Purpose of the conducted study was to evaluate the possibility of using underutilized seaweed (*Sargassum* sp.) to encapsulate prebiotics in dairy food applications. This will enable dairy based industries to utilize local materials instead of imports, whereas direct crude exports of the seaweeds can also be consumed for more valued added purposes. For encapsulation purposes, *Lactobacillus acidophilus* and *Bifidobacterium bifidum* strains were selected. When considering the surviving ability of the two strains of bacteria in 0.5% bile solution and gastric solution followed by intestinal juice and inclusive and non-inclusive of bile salt, it was evident that the strains coated with Sodium alginate from *Sargassum* provide a positive protection and therefore is foreseen as a better natural solution for probiotic encapsulation in dairy based food products. Extraction and utilization of local seaweeds in South Asia, has not been an exploited area so far, and therefore this research will form a base to formulate probiotics in local dairy industry by using *Sargassum* seaweed.

Keywords: alginate, coating, dairy-foods, probiotics, seaweeds

INTRODUCTION

Using the probiotics in dairy foods have increased rapidly in the past few decades, since majority of population seek health benefits from food. Live probiotics, when ingested or locally applied, tend to provide nutritional enhancements in the gut as well as the preventive effects of diarrohea caused by undesirable microorganisms in the gut **(Mallet *et al*, 1989)**. However, survivability of these probiotic microorganisms depends on many factors including; initial dosage and the cell viability maintained throughout the environment of the intestinal tract (**Gilliland, 1989**; **Mortazavian, 2006**). Therefore, in order to reach the acceptable level of the survivability, coating of the microorganism with a suitable substance is essential. By this mechanism, probiotics transported through the intestinal tract will survive throughout the passage of the upper digestive tract to bring about the necessary beneficial effect to the host. The technique used to coat the probiotic bacteria, is microencapsulation.

Microencapsulation is used in many food and non-food related applications with the advancement of sciences. The technology describes the process where the core material; the microbial cell; is retained in a matrix of the encapsulating shell matrix. Usage of this technique as a transport mechanism of probiotic bacteria, has enable to improve the survivability of the organism in gastrointestinal tract. Additionally, it has been used to improve the viability of the organisms in food products (**Rao & Shiwanaraian, 1989**). In many of the research-based applications, alginate is been used as the coating material due to the presence of its linear heteropolysaccharide; D-mannuronic and L-guluronic acids (**Smidsrod *et al*, 1972**). From earlier days, usage of alginate is favoured due to its simplicity, biocompatibility and affordability (**Martinsen *et al*, 1989**). Alginate gel is known to form a matrix with a pore size of less than 17 nm, (**Klein *et al*, 1983**) in which the probiotic cells with approximately 3µm in size will be trapped. Nevertheless, stability of these pure alginate gels is lesser, due to several factors. Presence of excessive amount of mono valent ions, Ca^{2+} chelating agents in the medium and severe environments are some of these factors (**Krasaekoopt *et al*, 2004**). To counter this matter, poly-cations and poly amino acids are been used (**Gombotz & Wee, 1998**) which forms strong complexes with alginate matrix and therefore is stable in afore mentioned adverse conditions. Therefore, alginates matrixed with poly cations are likely to improve the stability of the coating materials, resulting a better survivability of probiotics in the gut.

The intention of this study is to identify the influence of the alginate coating material when used as the carrier matrix of probiotic microorganisms, as well as its ability to survive in the gastrointestinal tract of individuals, until the final delivery location.

MATERIALS AND METHODS

Preparation of probiotics

Lactobacillus acidophilus slant and *Lactobacillus casei* (STI-12, CHR HANSEN PVT. LTD, Denmark) were inoculated with 10 ml of liquid MRS broth (SIGMA ALDRICH) separately and was incubated at 37°C for 24 hours at aerobic conditions. After incubation, cultures were transferred to 100 ml of MRS broth and the cells were centrifuged at 1500 rpm for 15 minutes (Kubota 4000) followed by washing twice repeatedly with 0.1% sterile peptone solution.

Extraction and preparation of sodium alginate solution from *Sargassum* sp.

About 20.0 g of the *Sargassum* sp. powder was mixed with 180 ml of 1M acetic acid and stirred at 80°C for 2 hours. The resultant solution was neutralized with 5M NaOH and was centrifuged at 1000 rpm for 10 minutes. Supernatant was collected.

Microencapsulation of probiotics

After obtaining the prepared probiotics, both cultures were mixed with 5ml of 0.1% sterile peptone solution and the liquid alginate solution prepared in the preceding step. The resultant was sterilized at 121°C for 15 minutes. The cell suspension was then injected in to 0.05M $CaCl_2$ solution and was allowed to stand for 20 minutes. After washing with 0.1% sterile peptone solution, formulated beads were kept at refrigerated conditions at 4°C.

For the beads prepared as per the above step, alginate and alginate-chitosan blend were applied as the coating materials. Coating was done with referring to the methods followed by **Zhou *et al* (1998).**

Coating probiotics with alginate

About 20.0 g of uncoated beads from the previous step were mixed with 200 ml of prepared and sterilized alginate solution. The mix was kept in the shaker at 100 rpm for 30 minutes and kept at the refrigerator in 4°C, after washing with peptone solution.

Coating probiotics with alginate-chitosan mix

1% (w/v) Chitosan (SIGMA-ALDRICH) was acidified with 0.4 ml of acetic acid glacial and 1M NaOH was added to adjust the pH between 5.8-6. The resultant was then filtered with Whatman filter paper and the final volume was made to 100 ml. The mix was then autoclaved at 121°C for 15 minutes. 20.0 g of refrigerated uncoated beads were then immersed in the prepared chitosan solution and was kept in the shaker at 100 rpm for 1 hour. Into this mix, sterilized alginate

solution was added and was shaken again at 100 rpm for 30 minutes. Finally, beads were washed with peptone solution, followed by the storage at 4°C.

Measuring the number of cells encapsulated

1g of uncoated beads and alginate coated beads were liquified with 1% Sodium citrate solution and shook gently for 10 minutes. 1g of chitosan beads were blended with a paddle blender for 1 minute followed by gentle shaking for 10 minutes. Resultant released probiotic bacteria were enumerated in MRS agar at 37°C for 72 hours. Finally, the cell count was captured for both treatments for both microorganisms.

Assessing the survivability of probiotics in bile salts

Formulated beads were kept in 0.6% bile solution (pH = 8) autoclaved at 121°C for 15 minutes and incubated at 37°C for 2 hours. Beads were removed from the incubator at 30 minute, 60 minutes, 90 minutes and 120 minutes time intervals. Finally, viable cells were enumerated in MRS agar at 37°C for 72 hours and counted separately for uncoated and differently coated beads.

Assessing the survivability of probiotics in stimulated gastric juice and intestinal juice without the presence of bile salts

Experiment was designed with the base from the previously conducted experiment by **Rao *et al* (1989)**. Sterile gastric juice was prepared by 0.08M HCl with 0.2% NaCl, pH; 1.5 and sterile intestinal juice was prepared by 0.05M KH_2PO_4, pH; 7.4 without bile salt.
About 2g of the prepared beads from different coating matrix combinations were placed in 20 ml of the gastric juice solution and incubated at 37°C for 30 minutes, 60 minutes, 90 minutes and 120 minutes respectively. This was followed by placing the same bead in the intestinal juice simulation and incubation was done at 37°C for 3 hours. After incubation, 2 ml of dissolved beads were enumerated in MRS agar at 37°C for 72 hours and analyzed.

Incorporation of probiotics to yoghurts and assessing the survivability across shelf life.

Yoghurt was prepared with a previously established recipe with having 10% of milk solids, 6% sugar, 2% yoghurt culture and 1% from the probiotics. After obtaining the results of the previous sub sections, probiotic variety with highest survivability of the respective matrix was selected for inclusion purposes. After inoculation of probiotics, count of the microorganisms was taken after certain time periods and was compared against that of the control yoghurts.
ANOVA was used for statistical analysis with the experimental design of factorial design.

RESULTS AND DISCUSSION

The cell count at the initial stage before the encapsulation process was in the range of 11.2 – 12.2 log cfu ml^{-1}. When considering the cell load after the encapsulation, the range for both coated and uncoated beads fell in between 11.3-11.5 log cfu g^{-1} beads. Difference between the coated and uncoated beads was not significant ($p>0.05$) and therefore implies that the coating methods used have not impacted the viability of cells. Therefore, the viable cell counts observed at different coating materials can be compared directly to the initial cell count of the un-encapsulated cells.
To assess the survivability of probiotics, destructive value (D-value) was used, which signifies the time required to destroy 90% or a single log cycle of the microorganism. D values obtained for the differently coated probiotics in the presence of the bile salt medium, are denoted in table I.

Table I Cell count after enumeration of the differently encapsulated probiotic bacteria, survived in the bile salt

Probiotics	Coating Material	0 Min.	30 Min.	60 Min.	90 Min.	120 Min.
Lactobacillus acidophilus	Uncoated	$2.2 \pm 0.06 \times 10^{9a}$	$2.2 \pm 0.06 \times 10^{7a}$	$7.2 \pm 0.06 \times 10^{5a}$	$4.8 \pm 0.06 \times 10^{4a}$	$3.2 \pm 0.06 \times 10^{3a}$
	Chitosan-Alginate	$1.2 \pm 0.06 \times 10^{9b}$	$1.4 \pm 0.06 \times 10^{8b}$	$1.3 \pm 0.06 \times 10^{7b}$	$4.2 \pm 0.00 \times 10^{6b}$	$3.3 \pm 0.06 \times 10^{5b}$
	Alginate	$1.0 \pm 0.06 \times 10^{9c}$	$1.2 \pm 0.1 \times 10^{8c}$	$1.2 \pm 0.06 \times 10^{7c}$	$3.5 \pm 0.12 \times 10^{6c}$	$4.3 \pm 0.12 \times 10^{5c}$
Lactobacillus casei	Uncoated	$4.6 \pm 0.14 \times 10^{7d}$	$3.9 \pm 0.1 \times 10^{6d}$	$4.1 \pm 0.21 \times 10^{5d}$	$4.0 \pm 0.10 \times 10^{5d}$	$1.1 \pm 0.12 \times 10^{4d}$
	Chitosan-Alginate	$8.3 \pm 1.07 \times 10^{8e}$	$6.7 \pm 0.08 \times 10^{7e}$	$5.8 \pm 0.15 \times 10^{6e}$	$3.7 \pm 0.31 \times 10^{6e}$	$8.2 \pm 0.21 \times 10^{5e}$
	Alginate	$7.9 \pm 1.25 \times 10^{7f}$	$5.1 \pm 0.1 \times 10^{6f}$	$3.9 \pm 0.08 \times 10^{6f}$	$1.1 \pm 0.00 \times 10^{6f}$	$7.9 \pm 1.10 \times 10^{5f}$

Mean ± Standard deviation from cell count; significant difference among columns were denoted by different superscripts ($p<0.05$) against control (uncoated). Means within the same column that have no common letters denote statistically significant differences among the figures concerned against the control.

When considering the initial stage after encapsulations, survival of *Lactobacillus acidophilus* ranged from $1.0 \pm 0.06 \times 10^9$ to $2.2 \pm 0.06 \times 10^9$. With the time of exposure to bile salts, survivability has decreased. Highest survivability after 120 minutes was observed in the alginate coated beads, closely followed by chitosan-alginate combined coating and finally, uncoated beads. However, until the last 30 minutes of observations, Chitosan-Alginate provided the best coating for probiotics. But, these values between alginate and chitosan-alginate combination did not depict a significant difference ($p<0.05$).

Table II Cell count after enumeration of the differently encapsulated probiotic bacteria, survived in the stimulated gastric juice and intestinal juice without the presence of bile salts

Probiotics	Coating Material	0 Min.	30 Min.	60 Min.	90 Min.	120 Min.
Lactobacillus acidophilus	Uncoated	$1.8 \pm 0.06 \times 10^{9a}$	$8.2 \pm 0.06 \times 10^{8a}$	$2.1 \pm 0.15 \times 10^{8a}$	$3.6 \pm 0.00 \times 10^{7a}$	$7.1 \pm 0.06 \times 10^{5a}$
	Chitosan-Alginate	$1.1 \pm 0.12 \times 10^{9b}$	$7.0 \pm 0.12 \times 10^{8b}$	$3.9 \pm 0.10 \times 10^{8b}$	$4.2 \pm 0.06 \times 10^{7b}$	$7.1 \pm 0.10 \times 10^{6b}$
	Alginate	$6.3 \pm 0.06 \times 10^{8c}$	$4.3 \pm 0.06 \times 10^{7c}$	$3.1 \pm 0.10 \times 10^{7c}$	$1.5 \pm 0.06 \times 10^{7c}$	$4.2 \pm 0.00 \times 10^{6c}$
Lactobacillus casei	Uncoated	$3.9 \pm 0.21 \times 10^{8d}$	$3.9 \pm 1.00 \times 10^{6d}$	$6.4 \pm 1.30 \times 10^{5d}$	$1.9 \pm 0.10 \times 10^{5d}$	$4.7 \pm 0.50 \times 10^{5d}$
	Chitosan-Alginate	$4.2 \pm 0.10 \times 10^{8e}$	$5.1 \pm 0.20 \times 10^{7e}$	$5.8 \pm 0.18 \times 10^{6e}$	$8.0 \pm 0.30 \times 10^{6e}$	$6.2 \pm 1.40 \times 10^{5e}$
	Alginate	$9.3 \pm 0.20 \times 10^{7f}$	$3.6 \pm 0.10 \times 10^{7f}$	$2.1 \pm 0.00 \times 10^{6f}$	$7.4 \pm 0.20 \times 10^{6f}$	$6.3 \pm 0.60 \times 10^{5f}$

Mean ± Standard deviation from cell count; significant difference among columns were denoted by different superscripts ($p<0.05$) against control (uncoated). Means within the same column that have no common letters denote statistically significant differences among the figures concerned against the control.

Lactobacillus casei survivability showed a significant difference event at the initiation of incubation($p<0.05$). Uncoated beads survived the least and the highest survivability is observed in Chitosan-Alginate combination, followed by Alginate coating with having a survivability of $8.2 \pm 0.21 \times 10^5$ and $7.9 \pm 1.10 \times 10^5$ respectively. The difference is significant ($p<0.05$).

When considering the survivability of the *L. acidophilus* in sequential incubation in stimulated gastric and intestinal juice without bile salts, highest survivability is observed in Chitosan-Alginate mix. Microencapsulated cells have survived better than the uncoated cells, where the initial cell count has ranged from $1.8 \pm 0.06 \times 10^9$ to $6.3 \pm 0.06 \times 10^8$. (Table II)

Although the survivability is higher in uncoated beads at the initial stage, this has overcome after 30th minute. In *L. casei* also, survivability in the gastric juice is highest with the Alginate-Chitosan combination with a significant difference of the results observed in uncoated and alginate coated beads.

With the identification of chitosan-alginate as the best coating material for both of *L. acidophilus* and *L. casei*, the coated beads were used to enrich yoghurt. Table III signifies the survivability data obtained after counted number of days.

As per the results in table 03, the control of both probiotics with uncoated bead has shown a drastic reduction of the count within the first 7 days of refrigeration with gradual decrease until the tested 21 days, and its significantly different ($p<0.05$) to the initial bacterial count. Also, from the day 01 to day 07, the reduction of the bacterial count is not significant ($p<0.05$). However, at initiation, the count of both probiotics coated with chitosan-alginate is significantly higher ($p<0.05$) than the uncoated beads.

Table III Survivability of encapsulate probiotics in the yoghurt medium (cfu/ml).

Probiotics	Coating Material	0 Days	7 days	14 days	21 days
Lactobacillus acidophilus	Uncoated	$4.3 \pm 0.12 \times 10^{9a}$	$5.9 \pm 0.01 \times 10^{7a}$	$7.2 \pm 0.02 \times 10^{5a}$	$1.9 \pm 0.03 \times 10^{4a}$
	Chitosan-Alginate	$5.4 \pm 0.02 \times 10^{9b}$	$6.8 \pm 0.20 \times 10^{8b}$	$5.7 \pm 0.15 \times 10^{6b}$	$1.3 \pm 0.09 \times 10^{5b}$
Lactobacillus casei	Uncoated	$5.7 \pm 0.10 \times 10^{9c}$	$1.5 \pm 0.02 \times 10^{8c}$	$6.1 \pm 1.2 \times 10^{5c}$	$2.0 \pm 0.15 \times 10^{5c}$
	Chitosan-Alginate	$6.3 \pm 0.28 \times 10^{9d}$	$7.4 \pm 0.05 \times 10^{8d}$	$7.6 \pm 0.20 \times 10^{6d}$	$9.3 \pm 0.01 \times 10^{5d}$

Mean ± Standard deviation from cell count; significant difference among columns were denoted by different superscripts ($p<0.05$) against control (uncoated). Means within the same column that have no common letters denote statistically significant differences among the figures concerned against the control.

When considering the survivability, *L. acidophilus* showed lesser ability *than L. casei*. Since the inoculation happened in equal amount of quantities of bacterium, this observation is justified. This maybe due to the ability of *L. casei* to survive in acidic medium, than *L. acidophilus.* After 7 days at refrigeration condition, highest count was observed in coated *L. casei* and was maintained until the 21st day.

For the stimulation study of the survivability in gastric juice, microorganisms were selected after careful consideration and background studies. According to **Sun & Griffith, 2000** and **Sultana *et al*, 2000,** it is evident that the survival rate of *Bifidobacterium Bifidum* was reduces drastically with time and will reach undetectable levels within 15 minutes. Another bacterium strain from *Bifidobacterium pseudolongum* has resisted in intestinal juice for 60 minutes but the survivability has reached zero afterwards (**Rao *et al*, 1989**). Therefore, Bifidobacterium strains are not selected for this study.

It has been identified in the previous studies also that the inclusion of chitosan into the microencapsulation process, as the coating material, will enhance an ion exchange reaction. Ion exchange process will absorb the bile salts and formulates a strong complex on the Chitosan-Alginate membrane. Once this strong insoluble complex is formed, it will prohibit the transfer of bile salts further and will protect the microbial cell inside (**Koo *et al*, 2001; Yu *et al*, 2001**) Therefore, it is evident that the microbe encapsulated in the alginate-chitosan mixed coat is surviving in the bile salt better than in the alginate coating. This identification is well justified with the study results as well, where the survivability is at its highest with Alginate-Chitosan coated bead.

However, it is also apparent that the survivability of the uncoated bead is higher in the gastric juice without bile salts, than the survivability of the alginate coated beads. This was similar to the observations recorded by **Sultana *et al*, 2000**, where its stated that the alginate does not protect the microbes and cannot withstand the highly acidic environments.

Considering the results obtained with the uncoated yoghurt inclusions, it is evident that the high acidity and the resultant low pH is not favourable for the *L. acidophilus* as well as *L. casei*. But *L. acidophilus* was more susceptible since the reduction of the population over time is higher than the other strain. This result is in parity to the study where L. acidophilus has shown a decrease of CFU from 2.57×10^7 to 5.01×10^5 over a period of 20 days (**Mortazavian *et al*, 2007**). Also, L. acidophilus count has reduced from 10^8 to 10^6 over 14 days of refrigerated conditions (**Kesenkas, 2010**).

Coated *L. acidophilus* showed significantly different results to uncoated probiotic. However, one experiment highlights that the L. acidophilus coated with alginate has better viability tested over 8 weeks at refrigerated temperature (**Shah *et al*, 1995**). However, since the first part of the study confirmed that the alginate-chitosan combination was a better coating material, it is evident that the survivability is better when tested with alginate-chitosan combination.

Assessing the survivability of *L. casei* has been a study of **Calleros *et al*, 2007**, where the coated probiotic has showed a better survivability than non-coated microorganism. By using alginate and a pectin combination, the study was able to convert the survivability from 86% to 96.38%.

CONCLUSION

Sargassum sp. seaweed extracted alginate can be used in combination with chitosan to improve the stability of probiotics. Out of the two probiotics tested, *L. casei* showed the better survivability in bile salts and *L. acidophilus* in gastric and intestinal juices without the presence of bile salts. This was from 2 Log_{10} and 1 log_{10} cycles respectively. As an inclusion for dairy based yoghurts, it is evident that the best solution will be to use chitosan-alginate coated *L. casei* as the inoculum, which will aid in the better survivability of the bacteria in the gut, thereby effecting positively on the gut health.

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