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Effect on Viability of Encapsulated Probiotic *Lactobacilli* Species Under Simulated Gastric and Intestinal Juice

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A B S T R A C T

Probiotics are one of the most common functional foods derived from milk and milk-based products. It contains microorganisms which ensure better activity of gastrointestinal tract. Dahi, lassi, sour milk, and yogurt are common probiotic products derived by fermentation. *Lactobacilli*, Bifidobacterium and *Bacilli* are among the most common microorganisms used for fermentation. Viability in the gastrointestinal tract is one of the major concerns about the development of probiotic products as the harsh conditions destroy potential microorganisms. Encapsulation of microorganisms with a suitable matrix can be a good approach to maintain viability. Starch, chitosan, alginate and vegetable oil are considered good matrices for encapsulation. In this study, encapsulated three Lactobacilli species were isolated from different sources. These species are *L. paracasei* MW561228, *L. plantarum* MW561227 and *L. plantarum* MW561230. Alginate, alginate/starch, alginate/chitosan and alginate/vegetable oil/tween 80 are used as encapsulated materials. 1.0%, 2.0% and 3.0% concentration of microorganisms are subjected to simulated gastric and intestinal conditions for up to 3 hours. Viability of cells is counted as CFU/ml to determine the effect on cell viability. Results of the study have shown that low concentrations of alginate/starch, alginate/chitosan and alginate/vegetable oil/tween 80 are very active even after exposure of 3 hours.

***Key words*:** Probiotics, *Lactobacilli species,* Encapsulation, Gastrointestinal tract, Viability

Probiotic is a term derived from the Greek word "probios" which means for life. It was actually introduced by Kollath in the year 1953. He has explained it as active substances that are essential for the healthy development of life [1-3]. Lilley and Stillwell [4] describe probiotics as substances secreted by microorganisms to stimulate the growth of other microorganisms [5-6]. Sperti [7] has described probiotics as tissue extracts that stimulate microbial growth [8-9]. Parker [10] has given a more clear definition mentioning a organisms and substances which contribute to intestinal microbial balance [11-12]. Fuller [13] (1989) gave an appropriate definition of probiotics as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance [14-16]. Finally, in the year 2001, the world health organization (WHO) defined probiotics as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host [17-19]. Elie Metchnikoff has suggested that, it may be feasible to remove harmful microbes with valuable microbes to modify the gut microbiota [20-21]. The very first probiotic microorganism was isolated from a breast fed infant by Henry Tissier. Initially, he gave the name *Bacillus bifidus communis*, which was renamed as *Bifidobacterium*. Later discoveries by Tissier have proved that *Bifidobacterium* is the prominent strain in the breast milk and helps the child to protect against diarrhea [22-23].

Probiotics have a wide application in the food industry. It is prepared using fermented milk and milk products like yogurts and curd. Supplementation of probiotics in food with a proper delivery system ensures betterment of gastrointestinal tract (GIT) activity [24-26]. The use of fermented dairy products is not a new concept. There are various products which were used in the past and are still being widely used. These products involve dahi, lassi, kefir, boruga, jemid, yogurt, sour milk, and kumys [27-30]. These products are prepared from various milk based raw materials in different countries. For example, lassi is one of the most popular drinks in India and Pakistan which utilizes cow, buffalo, goat and sheep milk, whereas boruga is produced in the Dominican Republic from whole cow milk [31-32].

The major issue with probiotic strains is their viability under extreme gastrointestinal conditions. Probiotics can only show good impact on health if they can reach to the intestine. Extremely acidic pH of the stomach destroys the probiotics and hence makes them useless. Encapsulation is one of the most studied approaches to maintain viability during harsh

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conditions. There are various matrices used for encapsulation depending on the type of microorganisms and application. Here, a study was carried out for the encapsulated three isolates obtained from milk samples. Four different encapsulation methods were applied and their impact on cell viability under simulated gastric and intestinal conditions was determined.

MATERIALS AND METHODS

Encapsulation with alginate

Calcium alginate was prepared in distilled water to obtain 1.0%, 2.0% and 3.0% alginate solution and sterilized by autoclaving at 110°C for 10 minutes. Alginate solutions were added to 0.1% of active culture cells. The mixture was filled into a sterile syringe with a narrow opening and beads were prepared by dropping the mixture from a height of around 15 cm into cold calcium chloride (0.5%). Beads were hardened for 30 minutes in the same mixture and then washed with a 0.85% NaCl solution to remove unbounded microorganisms. Beads were stored at 4°C until further use.

Encapsulation with alginate/starch

1.0%, 2.0% and 3.0% alginate and 2.0% starch was prepared and sterilized by autoclaving at 110°C for 10 minutes. 0.1% active culture was added to this sterile mixture and mixed well. The mixture was dropped into oil containing tween 80 (0.2%) and stirred vigorously for 20 minutes to form droplets. After 20 minutes, solution of 0.1 M calcium chloride was added from the side of wall of beaker and allowed to set for 30 minutes. After 30 minutes, the settled beads were collected and the oil drained. Beads were further washed twice with 0.85% saline and stored at 4°C until further use.

Encapsulation with chitosan

Low molecular weight chitosan was used. 0.4 gm of chitosan was dissolved in 90 mL of distilled water and 0.4 mL of glacial acetic acid was added for activation. After activation, pH was adjusted to 5.6 by 0.1 NaOH. The solution was filtered and autoclaved for sterilization after adjustment of the final volume to 100 mL. To this solution 10 gm of pre-prepared beads (with 1.0%, 2.0% and 3.0% alginate with active bacteria) were mixed and incubated for 50 minutes at very low speed. The resulted alginate-chitosan coated beads were washed with 1.0% peptone water and stored at 4°C until further use.

Encapsulation with vegetable oil and tween 80

Active bacterial culture containing alginate (1.0%, 2.0% and 3.0%), vegetable oil and tween 80 were mixed into the proportion of 3:3:0.5. The mixture was filled in a sterile syringe with a narrow opening and beads were made by dropping the mixture from a height of 15 cm into freeze cold solution of sodium chloride (0.5%) and calcium chloride (0.05%). Beads were allowed to harden for 30 minutes in the same mixture. After 30 minutes, it was washed with 0.85% NaCl solution to remove unbounded microorganisms. Beads were stored at 4°C until further use.

Determination of viability under simulated gastric condition

1.0% encapsulated and free active culture of probiotic microorganisms were inoculated into simulated gastric juice (NaCl 25 mM, KCl 7 mM, $NaHCO_3$ 45 mM, pepsin 3 g/L, pH 2.5) and allowed to incubate for 3 hrs. Aliquots were taken at regular intervals of 0 hr, 1 hr and 3 hrs and 50µL samples were spread on MRS agar and allowed to incubate for 24 hrs at 37°C under anaerobic condition. The total microbial count was calculated based on the sample taken for spreading and the results were noted in the form of log CFU/gm.

Determination of viability under simulated intestinal condition

1.0% encapsulated and free active culture of probiotic microorganisms were inoculated into simulated intestinal juice (NaCl 0.5% w/v, bile salt 0.5% w/v, pancreatin 1.0 g/L, pH 8.0) and allowed to incubate for 2 hrs. Aliquots were taken at regular intervals of 0 hr, 0.5hr, 1 hr, 1.5 hrs and 2.0 hrs and 50µL samples were spread on MRS agar and allowed to incubate for 24 hrs at 37°C. The total microbial count was calculated based on the sample taken for spreading and the results were noted in the form of log CFU/gm.

RESULTS AND DISCUSSION

Results obtained for the simulated gastric juice are mentioned in (Table 1-3, Fig 1-3). Results of *L. paracasei* MW561228 in simulated gastric juice have shown that as the concentration of alginate/chitosan (A/C) and alginate/vegetable/tween 80 (A/V/T) increases, the viability of cells also increases in the simulated gastric condition. 3.0% concentration of A/C have given highest 5.98±0.22 CFU/gm followed by 5.81±0.21 with a 2.0% concentration. In case of simulated intestinal condition, the highest viability was found with 2.0% concentration with 6.74±0.21 CFU/gm rather than 3.0% concentration. This may be because of the faster release of the cells from matrix. Among the two *L. plantarum*, MW561227 has shown better results as compared to MW561230. Similar to *L. paracasei* MW561228 optimum viability was seen with a 3.0% concentration of A/V/T with 5.65±0.28 CFU/gm in simulated gastric juice and 7.14±0.24 CFU/gm was seen in simulated intestinal condition with 2.0% concentration. Based on the overall results of viability, it was found that microorganisms encapsulated with 2.0% and 3.0% alginate have a better survival rate. Among all the combinations of encapsulation matrices, alginate/chitosan and alginate/vegetable oil/tween 80 have the higher stability in simulated gastric and intestinal conditions. There is no major viability difference obtained between 2.0% and 3.0% matrices. It was also observed that the 3.0 percent matrix shows slower release in simulated gastrointestinal conditions compared to the 2.0% matrix.

Table 1 Viability of 1.0% alginate encapsulated microorganisms under stimulated gastric juice condition

Microorganisms	Time (Hours)	Free	Alginate	A+S	A+C	A+V+T
Lactobacillus paracasei	0	6.21±0.24	5.99±0.19	6.19±0.19	6.2±0.12	6.08±0.14
MW561228	1	5.48±0.29	5.19±0.13	5.46±0.2	5.43±0.24	5.45±0.23
	3	3.54±0.23	4.44±0.26	4.78±0.21	4.94±0.18	4.84±0.16
Lactobacillus plantarum	0	6.27±0.2	5.97±0.19	6.15±0.22	6.04±0.11	6.08±0.23
MW561227	1	5.22±0.15	5.63±0.19	5.36±0.16	5.76±0.2	5.75±0.18
	3	3.54±0.16	4.79±0.19	4.78±0.27	4.83±0.25	4.92±0.2
Lactobacillus plantarum	0	6.47±0.19	6.38±0.2	6.14±0.1	6.17±0.2	6.34±0.28
MW561230	1	5.47±0.27	5.3±0.27	5.52±0.46	5.32±0.26	5.31±0.15
	3	3.47±0.19	4.54±0.41	4.6±0.12	4.66±0.3	4.67±0.23

Table 2 Viability of 2.0% alginate encapsulated microorganisms under stimulated gastric juice condition

Microorganisms	Time (Hours)	Free	Alginate	A+S	A+C	A+V+T
Lactobacillus paracasei	0	7.31±0.28	7.05±0.22	7.28±0.22	7.29±0.14	7.15±0.16
MW561228	1	6.45±0.34	6.11±0.15	6.42±0.24	6.39±0.28	6.41±0.27
	3	5.17±0.27	5.22±0.31	5.62±0.25	5.81±0.21	5.69±0.19
Lactobacillus plantarum	0	7.38±0.24	7.02±0.22	7.24±0.26	7.11±0.13	7.15±0.27
MW561227	1	6.14±0.18	6.62±0.22	6.31±0.19	6.78±0.23	6.77±0.21
	3	5.16±0.19	5.64±0.22	5.62±0.32	5.68±0.29	5.79±0.24
Lactobacillus plantarum	0	7.61±0.22	7.51±0.24	7.22±0.12	7.26±0.24	7.46±0.33
MW561230	1	6.43±0.32	6.23±0.32	6.49±0.54	6.26±0.31	6.25±0.18
	3	5.08±0.22	5.34±0.48	5.41±0.14	5.48±0.35	5.49±0.27

*A+S = Alginate + Starch, A+C = Alginate + Chitosan, A+V+T = Alginate + Vegetable oil + Tween 80

Table 3 Viability of 3.0% alginate encapsulated microorganisms under stimulated gastric juice condition

Microorganisms	Time (Hours)	Free	Alginate	A+S	A+C	A+V+T
Lactobacillus paracasei	0	7.53±0.29	7.26±0.23	7.5±0.23	7.51±0.14	7.36±0.16
MW561228	1	6.64±0.35	6.29±0.15	6.61±0.25	6.58±0.29	6.6±0.28
	3	4.3±0.28	5.38±0.32	5.79±0.26	5.98±0.22	5.86±0.2
Lactobacillus plantarum	0	7.6±0.25	7.23±0.23	7.46±0.27	7.32±0.13	7.36±0.28
MW561227	1	6.32±0.19	6.82±0.23	6.5±0.2	6.98±0.24	6.97±0.22
	3	4.28±0.2	5.81±0.23	5.79±0.33	5.85±0.3	5.96±0.25
Lactobacillus plantarum	0	7.84±0.23	7.74±0.25	7.44±0.12	7.48±0.25	7.68±0.34
MW561230	1	6.62±0.33	6.42±0.33	6.68±0.56	6.45±0.32	6.44±0.19
	3	4.2±0.23	5.5±0.49	5.57±0.14	5.64±0.36	5.65±0.28

*A+S = Alginate + Starch, A+C = Alginate + Chitosan, A+V+T = Alginate + Vegetable oil + Tween 80

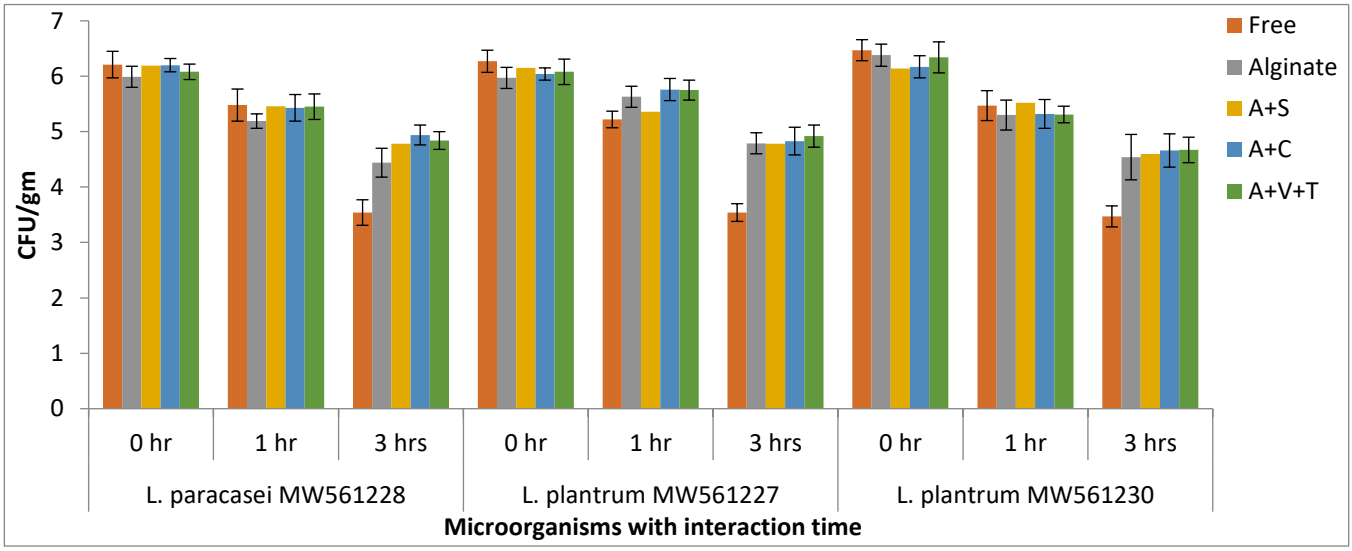

Fig 1 Viability of 1.0% alginate encapsulated microorganisms under stimulated gastric juice condition

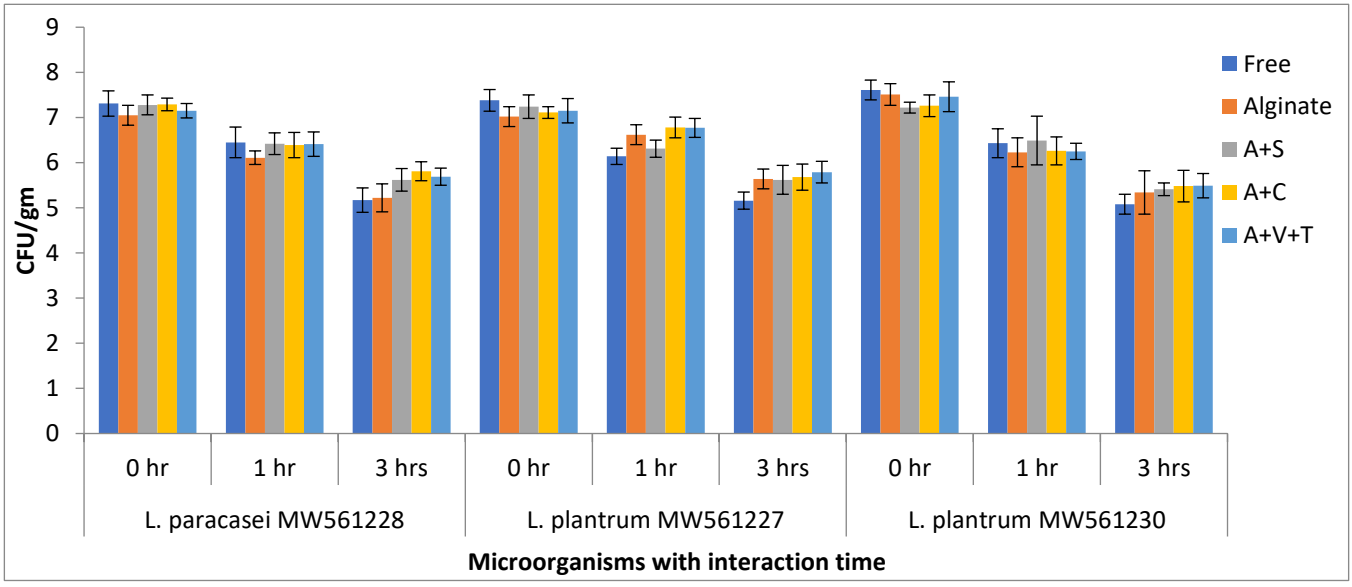

Fig 2 Viability of 2.0% alginate encapsulated microorganisms under stimulated gastric juice condition

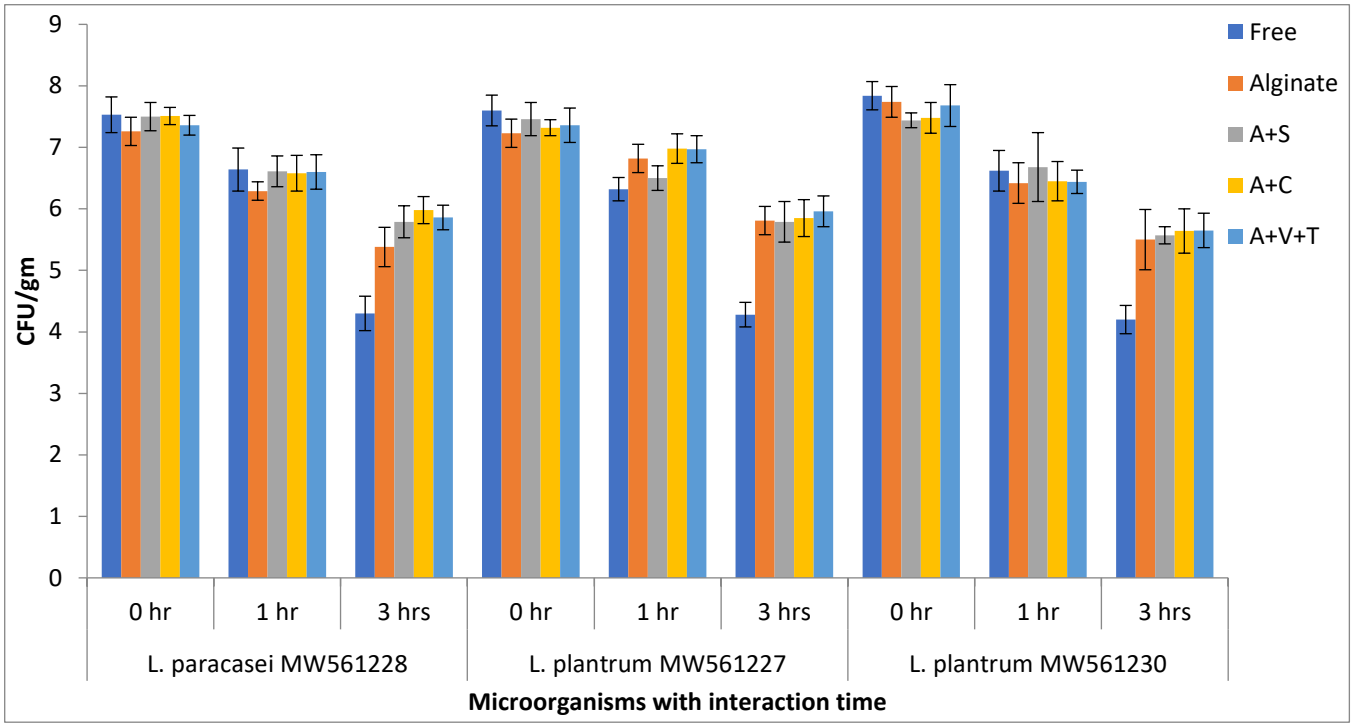

Fig 3 Viability of 3.0% alginate encapsulated microorganisms under stimulated gastric juice condition

It was seen that alginate and gelatin were among the most common matrices used for encapsulation, but compare to other matrices they were weak and not suitable for long term storage [33-35]. Studies have suggested that the addition of other compounds can increase the binding efficiency and can also enhance the viability for a longer period of time [36-38]. Chitosan was proved to be one of such compounds which has provided maximum stability to encapsulated microorganisms [39]. In a study by Zanjani *et al.* [40] microorganisms encapsulated with calcium alginate with starch and chitosan showed very high resistance under adverse condition of the GI tract. Their study have shown more than 96% survival rate of *L. casei* and *B. bifidum* even after 2 hrs of exposure. Chitosan-alginate nanoparticles are also used for controlled release of vitamin B2 and drugs like nifedipine [41-42]. Caetano *et al.* [43] have shown that encapsulation of BCG with alginate/chitosan can be effectively used for the intranasal route.

The (Tables 4-6) contains the results obtained for the simulated intestinal conditions. Based on the overall results, it was seen that in the initial phase of up to 1 hour, good growth was observed in free (uncapsulated) microorganisms. But as time passes and reaches to 3 hours, the viable count of uncapsulated microorganisms were decrease and all the encapsulated have shown good growth. 1.0% and 2.0% have comparatively shown good growth as compared to 3.0%. The principal reason behind this is the low rate of release of microorganisms [44-46].

Table 4 Viability of 1.0% alginate encapsulated microorganisms under various conditions of simulated intestinal juice

Microorganisms	Time (Hours)	Free	Alginate	A+S	A+C	A+V+T
Lactobacillus paracasei	0	6.58±0.25	4.98±0.21	5.02±0.23	5.34±0.13	5.36±0.15
MW561228	1	5.75±0.31	5.13±0.14	5.67±0.22	5.89±0.25	5.98±0.25
	3	5.13±0.25	5.21±0.28	5.76±0.23	6.13±0.19	6.25±0.17
Lactobacillus plantarum	0	6.84±0.22	5.18±0.19	5.22±0.24	5.56±0.12	5.57±0.25
MW561227	1	5.98±0.16	5.34±0.21	5.9±0.17	6.12±0.21	6.22±0.19
	3	5.34±0.17	5.41±0.22	5.99±0.29	6.38±0.26	6.5±0.22
Lactobacillus plantarum	0	6.65±0.18	5.03±0.22	5.07±0.11	5.40±0.22	5.41±0.28
MW561230	1	5.81±0.29	5.18±0.29	5.73±0.49	5.95±0.28	6.04±0.16
	3	5.18±0.16	5.26±0.44	5.82±0.13	6.19±0.32	6.31±0.25

*A+S = Alginate + Starch, A+C = Alginate + Chitosan, A+V+T = Alginate + Vegetable oil + Tween 80

Table 5 Viability of 2.0% alginate encapsulated microorganisms under various conditions of simulated intestinal juice

Microorganisms	Time (Hours)	Free	Alginate	A+S	A+C	A+V+T
Lactobacillus paracasei	0	7.23±0.28	5.47±0.22	5.52±0.22	5.87±0.14	5.89±0.16
MW561228	1	6.32±0.28	5.64±0.22	6.23±0.22	6.47±0.14	6.57±0.16
	3	5.64±0.27	5.72±0.31	6.33±0.25	6.74±0.21	6.87±0.19
Lactobacillus plantarum	0	7.52±0.24	5.69±0.22	5.74±0.26	6.1±0.13	6.13±0.27
MW561227	1	6.57±0.18	5.87±0.22	6.48±0.19	6.73±0.23	6.83±0.21
	3	5.87±0.19	5.95±0.22	6.58±0.32	7.01±0.29	7.14±0.24
Lactobacillus plantarum	0	7.30±0.22	5.52±0.24	5.58±0.12	5.93±0.24	5.95±0.33
MW561230	1	6.38±0.32	5.70±0.32	6.29±0.54	6.53±0.31	6.64±0.18
	3	5.70±0.22	5.78±0.48	6.39±0.14	6.81±0.35	6.94±0.27

*A+S = Alginate + Starch, A+C = Alginate + Chitosan, A+V+T = Alginate + Vegetable oil + Tween 80

Table 6 Viability of 3.0% alginate encapsulated microorganisms under various conditions of simulated intestinal juice

Microorganisms	Time (Hours)	Free	Alginate	A+S	A+C	A+V+T
Lactobacillus paracasei MW561228	0	7.19±0.27	6.94±0.21	7.16±0.21	7.17±0.14	7.04±0.16
	1	6.35±0.33	6.01±0.15	6.32±0.23	6.29±0.27	6.31±0.26
	3	4.10±0.26	5.14±0.30	5.53±0.24	5.72±0.21	5.60±0.19
Lactobacillus plantarum MW561227	0	7.26±0.23	6.91±0.21	7.12±0.25	7.01±0.13	7.04±0.26
	1	6.04±0.18	6.51±0.21	6.21±0.19	6.67±0.22	6.66±0.21
	3	4.09±0.19	5.55±0.21	5.53±0.31	5.59±0.28	5.70±0.23
Lactobacillus plantarum MW561230	0	7.49±0.21	7.39±0.23	7.10±0.12	7.14±0.23	7.34±0.32
	1	6.33±0.31	6.13±0.31	6.39±0.53	6.16±0.3	6.15±0.18
	3	4.01±0.21	5.25±0.47	5.32±0.14	5.39±0.34	5.40±0.26

*A+S = Alginate + Starch, A+C = Alginate + Chitosan, A+V+T = Alginate + Vegetable oil + Tween 80

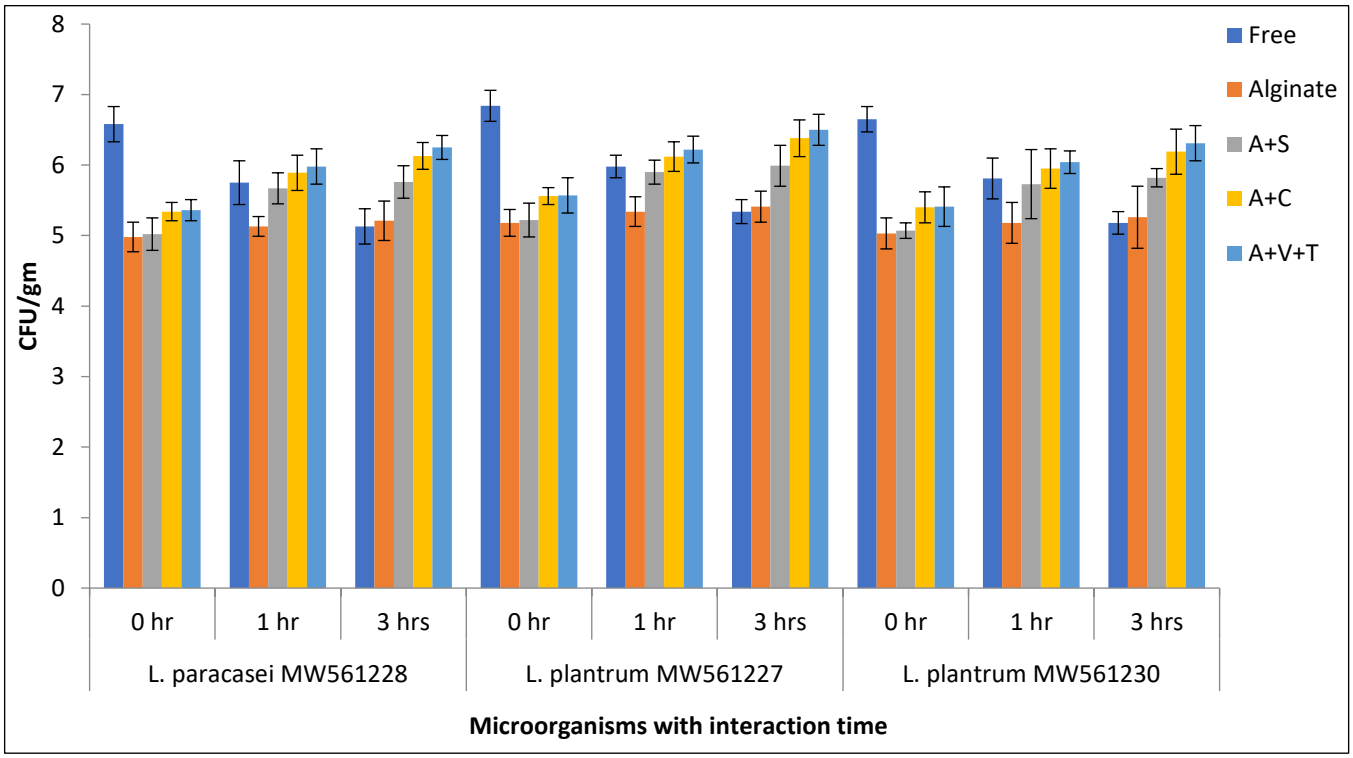

Fig 4 Viability of 1.0% alginate encapsulated microorganisms under various conditions of simulated intestinal juice

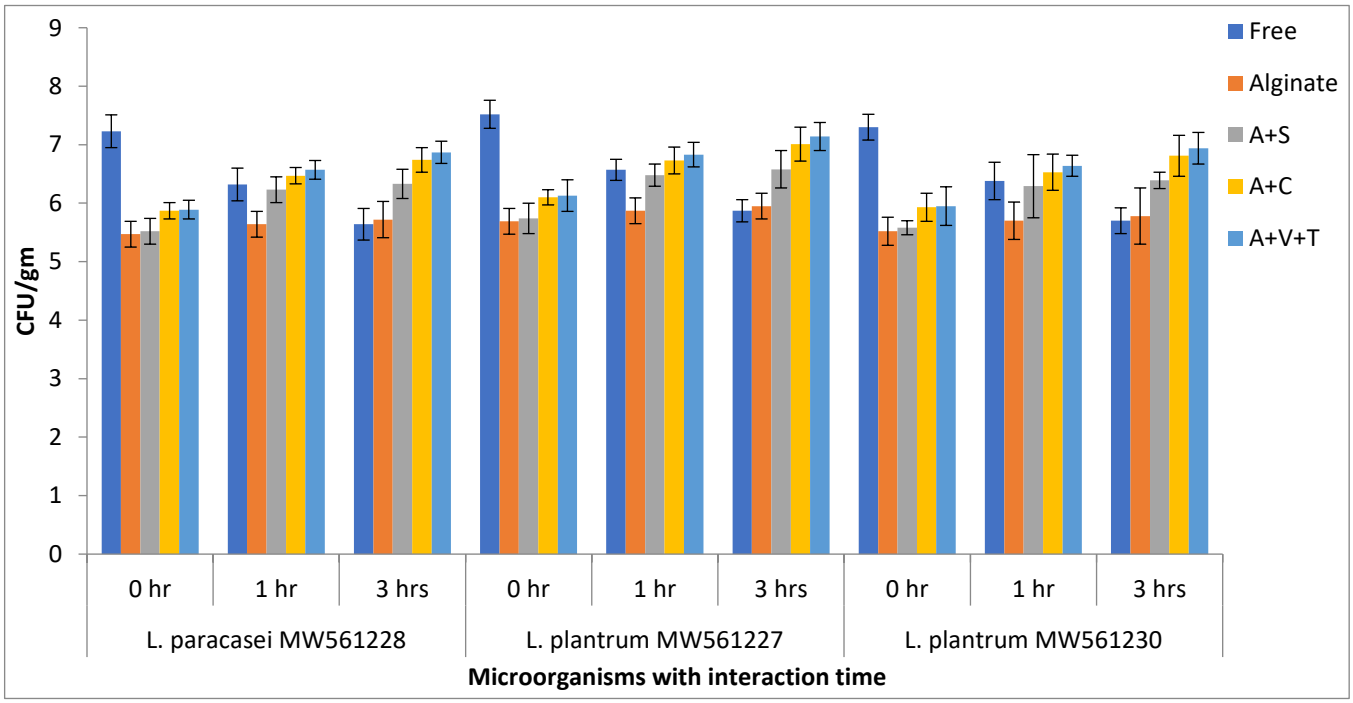

Fig 5 Viability of 2.0% alginate encapsulated microorganisms under various conditions of simulated intestinal juice

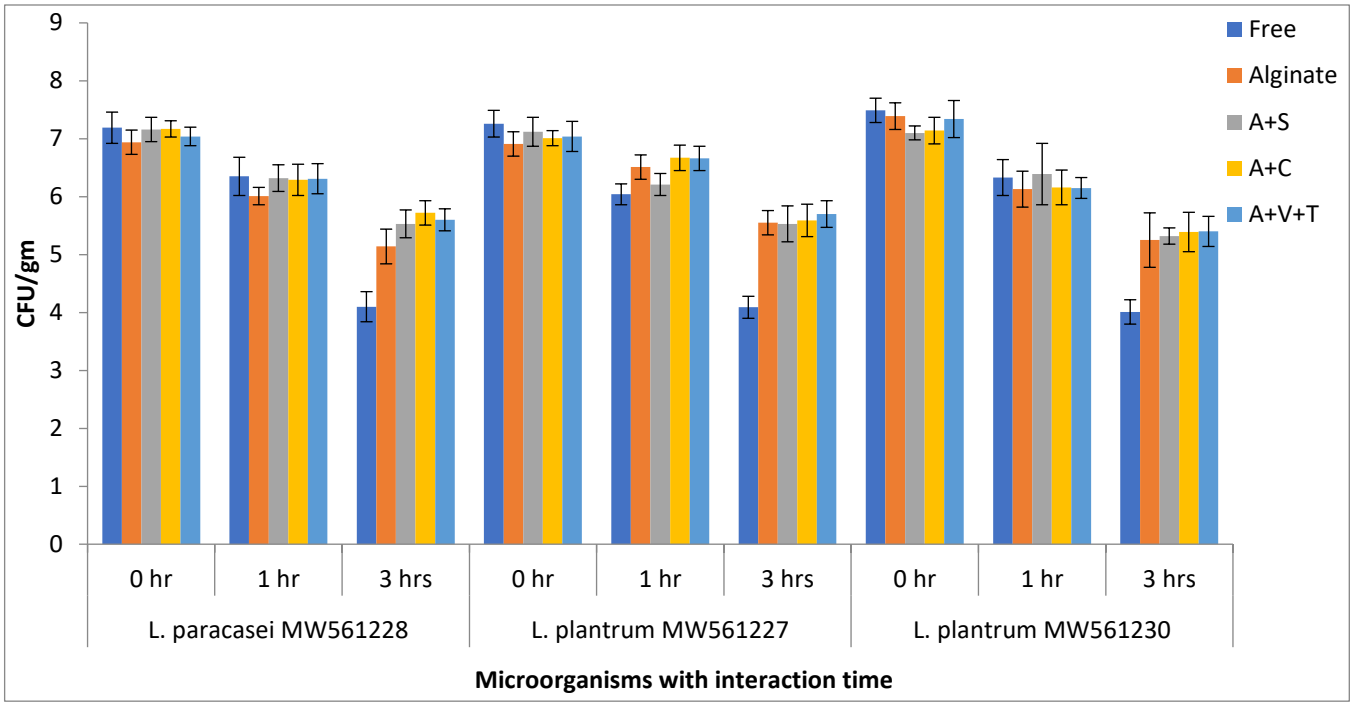

Fig 6 Viability of 3.0% alginate encapsulated microorganisms under various conditions of simulated intestinal juice

Since the pH of simulated intestinal fluid is around 8.0%, it won't inhibit the growth of microorganisms significantly. Ayama *et al.* [47] have found that encapsulation with starch and alginate enhances the viability of cells under simulated intestinal conditions. A similar observation was made by Sultana *et al.* [48] during their study with Lactobacilli and Bifidobacterium. Not limited to gastrointestinal conditions, they have also determined the viability of encapsulated microorganisms in yogurt. Based on the results of their study, they have found that bead size affects significantly on the viability hence it must be optimized for better viability. Samedi and Charles have proved that the addition of maltodextrin with starch provided better stability and allowed the microorganisms to reach the large intestine [49]. In a study carried out on *L. plantarum* by Preapanitchai *et al.* [50] they found that hydro gel beads prepared using calcium, alginate and soy protein are stable at temperature up to 72°C with under extreme acidic conditions of pH 2. They have also used these hydrogel beads for mango juice fortification. Upon pasteurization, the viability of *L. plantarum* was retained significantly. Lulwah *et al.* [51] have determined the effects of various encapsulation materials on *L. plantarum* DSM 20174. The encapsulation material involved a combination of alginate with sodium chloride, canola oil, olive oil and chitosan. Results of their study have shown that, olive oil capsules have provided maximum stability at pH 2 even after incubating for 24 hrs. The addition of bile salt up to concentration of 0.5% has enhanced the stability of capsules prepared using chitosan and olive oil. The combination of sodium chloride and chitosan had given highest stability at higher temperature as compared to other combinations. Based on the overall study, they have recommended using chitosan with NaCl for long term stability of probiotic strains. Jimenez-Fernandez *et al.* [52] tried gum Arabic and a pectin mixture for encapsulation of *L. paracasei* and studied various physico-chemical parameters. In the study, they found that size of the microcapsule can greatly influence the texture, quality and sensory properties of the product. No doubt, encapsulation with gum arabic and pectin has protected *L. paracasei* against the harsh conditions of simulated gastrointestinal conditions. Ortakci *et al.* [53] have surprisingly found that simulated gastric juice with hydrochloric acid can significantly affect the viability of encapsulated *L. paracasei*. They have also observed that alginate encapsulated strains have better survival in the presence of phosphoric acid. However, the reason behind this is still not known. LBC-1e for retention of viability, encapsulation is a better option but for long term preservation, lyophilization is considered as best approach. Studies have shown that lyophilized strains can be stored for many months without losing viability. One such study was carried out by Jofre *et al.* [54] to determine the effect of various cryoprotectants on the viability of *L. paracasei* strains. They have used glucose, trehalose, skim milk and lacrosse either alone or in combination as cryoprotectants. They have found that storage of lyophilized cells at 4°C maintain higher stability and viability of cells. When they are stored at 22°C, it was found that glucose and combinations of glucose are not efficient cryoprotectants.

Based on results obtained in this study, it was found that careful selection of encapsulation matrices is very important to protect probiotic microorganisms against harsh condition of digestive tract. Now a day wide range of encapsulation matrices are available for encapsulation. Selection of suitable matrix is highly depends on the type of probiotic microorganisms and its applications. Any single matrix or combination of more than one matrix can be used at a time. For each study, it is recommended to optimize all the relevant parameters for better outcome of the study [55-60].

CONCLUSION

Microencapsulation has a lot of advantages in the food industry as it solves the problem of probiotics in food products having poor viability. Ideally, the viability of beneficial probiotic bacteria should be kept at the standard level required, which is one of the conditions for using microorganisms as dietary supplements in the carrier food before being consumed. In this study, *Lactobacillus paracasei* MW561228, *Lactobacillus plantarum* MW561227 *and Lactobacillus plantatum* MW561230 probiotic bacteria were encapsulated within different wall materials including alginate, alginate/starch, alginate/chitosan and alginate/vegetable oil/tween 80 through the extrusion/emulsion method to improve

their survivability in gastrointestinal conditions and long-term storage. The viability of the isolates was examined in simulated gastrointestinal juice. It was found that microorganisms encapsulated with 2.0% and 3.0% alginate have a better survival rate with alginate/chitosan and alginate / vegetable oil / tween80 matrix in simulated gastric and intestinal conditions. There is no significant difference found in the viability between 2.0% and 3.0% matrices, so it is advisable to use 2.0% alginate instead of 3.0% alginate as it will not easily release the microorganisms.

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