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RESEARCH ARTICLE

SYNBIOTIFICATION OF FRUIT JUICES BY MICROENCAPSULATED LACTIC ACID BACTERIA STRAINS

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ABSTRACT

Probiotics are "live micro-organisms which, when administered in adequate amounts, confer a health bene t on the host". For the present study three different strains of Lactic acid Bacteria were procured from NCIM, Pune and Dairy Science College, Bangalore. The strains include *L. casei*, *L. brevis* and *L. lactis*. These LAB strains were co-microencapsulated with the prebiotics to form synbiotic food products. These synbiotics formed with highly efficient strains of probiotics and are highly bioactive. Prebiotics are the non digestible food ingredient that beneficially affects the host by selectively stimulating the growth of bacteria in the colon. The growth of LAB was studied by turbidometric measurements in the presence and absence of prebiotics at 48h and 72h of incubation. The prebiotics like pectin, oats and a combination of both was seen to increase the growth of the different strain of LAB. Co-microencapsulation of the synbiotic strains were done using Sodium Alginate followed by inoculating them into different fruit juices (grape, watermelon, mosambi, and pine apple) for fermentation. The synbioticated substrates were evaluated for pH and turbidity and were tested for shelf life. The pH level decreased with the increase in acid production in the fermented juices, the highest was observed in synbiotic grape juices. This was followed by increase in turbidity of the samples. The viability of the immobilized synbiotic cultures remained for a longer period of time. Understanding the synbiotic phenomena between the strains is of prime importance for the control of the cultures and could be used for developing a functionally healthy beverage or functional food to promote health and nutrition of the consumer. Prospective studies on mechanisms of the probiotic activities may enable their new medical applications for lactose intolerant and diabetic patients.

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INTRODUCTION

Probiotics are "The viable microbial food supplement which beneficially influences the health of the host" (Schrezenmeir and de Vrese). The strains of probiotics used are *L.lactis, L.casei* and *L.brevis.* Probiotics have specific requirements i.e, the environment should be fermentative, aerotolerant or anaerobic, and aciduric or acidophilic (Marimuthu *et al*., 2014). Probiotics showed significant growth when supplemented with the prebiotics in the MRS media. Prebiotics are the "indigestible fermented food substrates that selectively stimulate the growth, composition, and activity of microflora in gastrointestinal tract and thus improve host's health and well-being". Prebiotics such as pectin, oats and a mix of both in equal ratios was used. When probiotics and prebiotics are used in combination, they are known as "synbiotics." The combination of suitable probiotics and prebiotics enhances survival and activity of the organism. These synbiotic product was used to ferment the fruit juices to form a functional food. The term 'functional' is sometimes used to describe foods and drinks that are enriched with particular nutrients or substances that have the potential to positively influence health over and above their basic nutritional value. A functional ingredient can be defined as a dietary ingredient that affects its host in a targeted manner so as to exert positive effects that justify certain health claims The current study aims at encapsulation of the probiotic bacterial strains in alginate beads along with the prebiotics to protect the probiotics from the secondary metabolites including anti microbial compounds such as formic acid, benzoic acid, hydrogen peroxide and bacteriocin present in the fruit juices This will lead to the increase in the viability of the bacterial cells. . This process focuses on aspects of delivery and the potential use, where two or more bioactive ingredients can be combined to have a synergistic effect. (Claude P Champagne and Patrick Fustier, 2007)

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MATERIALS AND METHODS

Growth and Maintenance of Probiotic Bacteria

The different strains of lactic acid bacteria like *Lactobacillus lactis* (NCIM 2368) and *Lactobacillus brevis* (NCIM 2090) was used for the study were procured from NCIM, Pune (National Collection of Industrial Microorganisms). *Lactobacillus casei* was obtained from Dairy Science College, Bangalore. The strains were selected based on their fermentation capacity. The bacteria were subcultured on a weekly basis. These were grown in selective media MRS medium (de Man, Rogosa and Sharpe). About 2% of starter culture was inoculated into MRS broth aseptically and was incubated in shaker at 37^{o}C for 24h and then stored at 4^{o}C until used.

Effect of different prebiotics on the Probiotic LAB strains: (Emon *et al.*, 2015)

Prebiotics were added in the MRS broth to monitor the effect of the prebiotics on the growth rate of the probiotic bacterial strains. In this study commercial sample of oats and pectin (Sigma-Aldrich) were being used to study the growth pattern of the Probiotics. The sub-cultured LAB strains were inoculated freshly in sterile MRS broth. About 5% of inoculum of all the strains was inoculated in autoclaved MRS broth and incubated at 37^{o}C in shaker at 60rpm. The growth was checked at 24h and 48h using UV Vis Spectrophotometer at 660nm using uninoculated MRS broth as control. Further the growth of probiotics was studied in the presence of prebiotics. To the MRS broth 5% inoculum (LAB strains) and 0.4% of the prebiotics (Singly or both) were added. The growth was checked at 24h and 48h using UV-Vis Spectrophotometer at 660nm. The above mentioned sets were incubated in shaker at 37^{o}C at 60rpm. The optical density readings were taken at 24h and 48h intervals using UV Vis spectrophotometer. About 2mL of sterile broth was used to set the reference followed by 2mL of the media was being used to check the O.D. Comparative studies of the growth pattern were done of the LAB with and without prebiotics

Encapsulation and Co-microencapsulation: (Gaanappriya *et al.*, 2013)

Lactic acid bacteria which were previously grown were subjected to immobilization. The LAB strains (5mL) were aseptically added in 2% Sodium Alginate (5mL) and mixed well. This mix was added drop wise in 0.33M CaCl2 (25mL) for jellification to form beads. The above mix was incubated at room temperature at 26^{o}C for an hour in sterile atmosphere in the laminar flow hood to solidify and harden. The immobilized beads were then stored in 10mL of autoclaved MRS broth at 4 ^{o}C. The LAB strains grown in the presence of prebiotics were subjected to immobilization. The samples showing higher growth rate were being selected and subjected to co-microencapsulation. The LAB strains (5mL) were aseptically added in 2% Sodium Alginate (5mL) and mixed well. This mix was added drop wise in 0.33M $CaCl_2$ (25mL) for jellification to form beads. The above mix was incubated at room temperature at 26^{o}C for an hour in sterile atmosphere to solidify and harden. The immobilized beads were then stored in 10mL of autoclaved MRS broth at 4^{o}C

Synbiotification of fruit juices

Preparation of the Substrates

The fruit used in the present study include *Vinis vitifera* (c.v.grapes), *Citrus limetta* (c.v. Mosambi), *Ananus comosus* (c.v.pineapple) and *Citrullus lanatus* (c.v. watermelon) are purchased from the local market. The selected fruits were washed thoroughly with running tap water, rinsed with distilled water and blotted dry. The seeds are separated manually from the pulp. The juice was then extracted and strained using the double fold Muslin cloth. These juices were sterilized and heated and used as substrates for further studies. Initial O.D values and pH of the juices were noted down.

Inoculation of the co-encapsulated strains into the substrates

The previously stored beads were decanted off the broth and inoculated in the substrates. 2% of the inoculum of all strains and the co-microencapsulated beads were inoculated into 40mL of all fruit juices at room temperature 26 ^{o}C aseptically. These were incubated at 37^{o}C in the shaker at 60rpm and allowed for fermentation process. The optical density values were being checked at 48h and 72h of incubation using UV Vis spectrophotometer at 540nm. 2mL of distilled water is used to set the reference.

Titratable acidity

The titratable acidity of the synbiotificated fruit juices was determined titrimetrically using 0.1M NaOH and 0.1M Phenolphthalein as indicator. About 5mL of juice was pipette out into 50mL conical flask, to this 6-7 drops of indicator was added and titrated against NaOH (50mL) in burette. The end point was noted as the colour change in the sample. Three trials were done to get concordant values.

Shelf life study

The synbiotificated fruit juices after 72h were stored at 4^{o}C for 8 weeks. Samples were checked for its turbidity on weekly basis. For this 2mL of the juice was used to check the O.D at 540nm using UV Vis spectrophotometer.

RESULTS

Growth of Probiotic LAB strains in the absence and presence of prebiotics

The bacteria were exposed to different prebiotic samples in order to ascertain whether the exposure to prebiotics would enhance the growth characteristics of these organisms.

Growth of LAB strains

The growth of LAB strains was studied by turbidometric measurements. The LAB strains were inoculated in MRS broth at 37ºC and their optical density were taken after 48h and 72h of incubation. Significant growth was observed in *L. casei* and minimum growth was observed in *L. lactis*. The results were tabulated in Table 2 and Figure 1.

Table 1. Growth of LAB strains in absence and presence of Prebiotics by Turbidometric measurements

LAB Strains	In absence of Prebiotics (mean±standard deviation)		In Presence of Prebiotics (mean±standard deviation)					
			with Oats		with Pectin		with Oats and Pectin	
	O.D. (660nm) at 48h	O.D. (660nm) at 72h	O.D. (660nm) at 48h	O.D. (660nm) at 72h	O.D. (660nm) at 48h	O.D. (660nm) at 72h	O.D. (660nm) at 48h	O.D. (660nm) at 72h
L.casei	1.009±0.15	2.237±0.15	1.377±0.13	2.526±0.13	1.1±0.12	2.285±0.12	1.11±0.12	2.451±0.12
L.lactis	0.797±0.15	1.548±0.15	2.124±0.15	2.2±0.15	1.311±0.18	1.423±0.18	1.939±0.18	1.99±0.18
L.brevis	1.489±0.13	1.782±0.13	2.092±0.16	2.1±0.16	1.523±0.22	1.782±0.22	1.36±0.22	1.97±0.22

Table 2. Turbidometric measurements and pH of substrates

Substrates	O.D. at 540nm	pH
Vitis vinifera	0.148	4.12
Ananas comosus	0.030	4.90
Citrus limetta	0.048	4.80
Citrullus lanatus	0.120	5.35

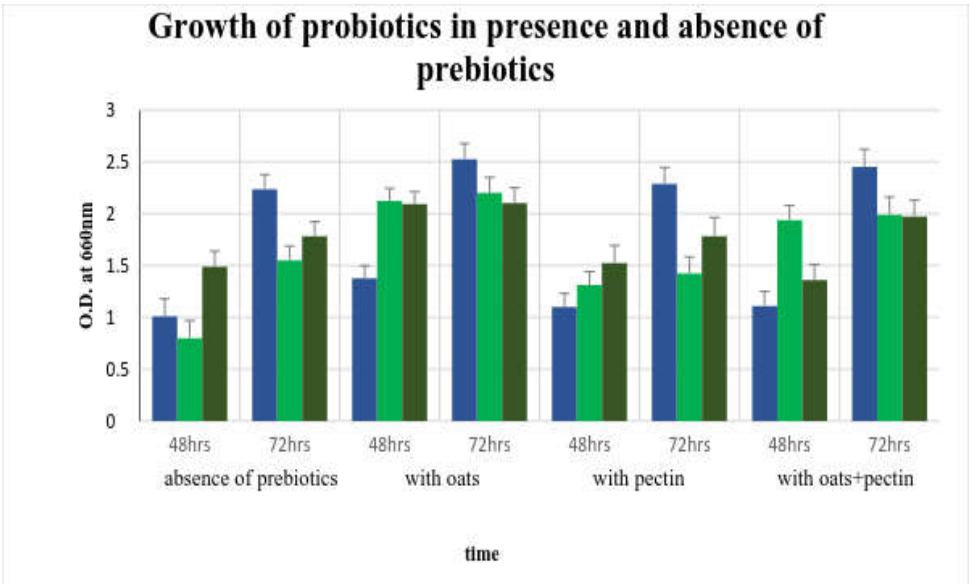

Figure 1. Growth of LAB strains in absence and presence of Prebiotics by Turbidometric measurements

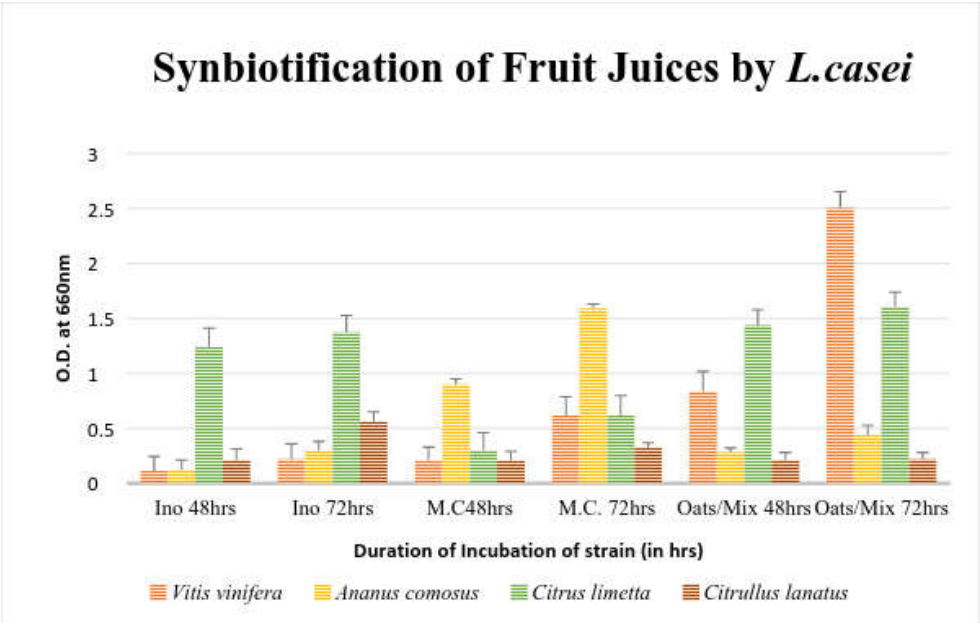

Figure 2. Synbiotic potential of Fruit juices and synbiotification by *L.casei*

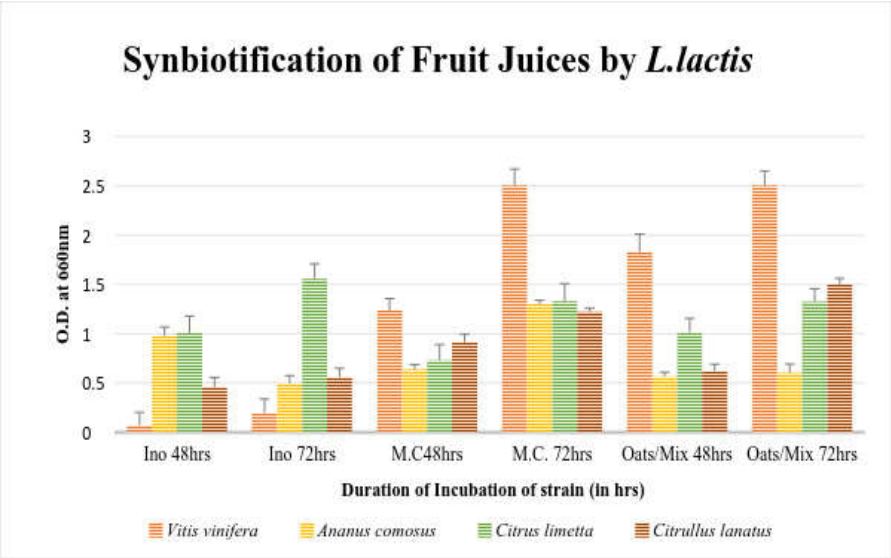

Figure 3. Synbiotic potential of Fruit juices and synbiotification by *L.lactis*

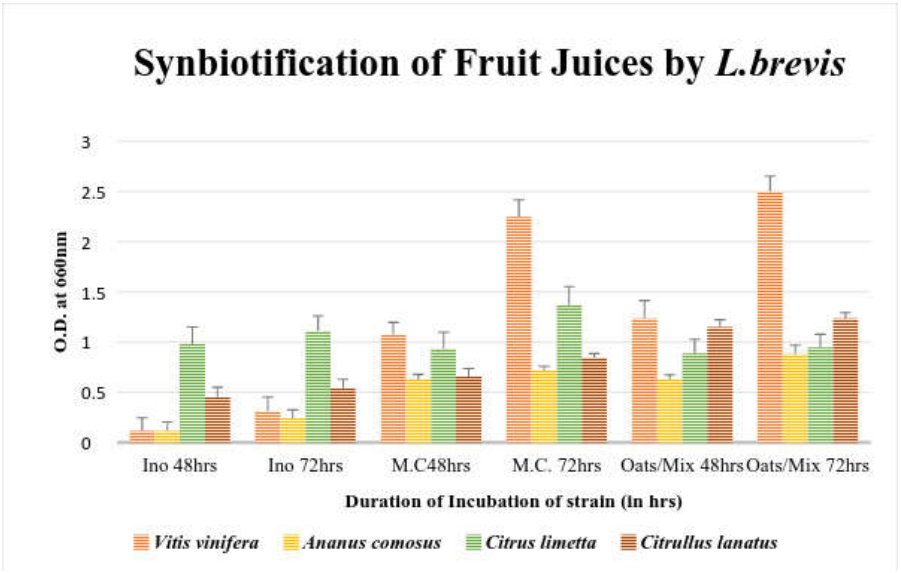

Figure 4. Synbiotic potential of Fruit juices and synbiotification by *L.brevis*

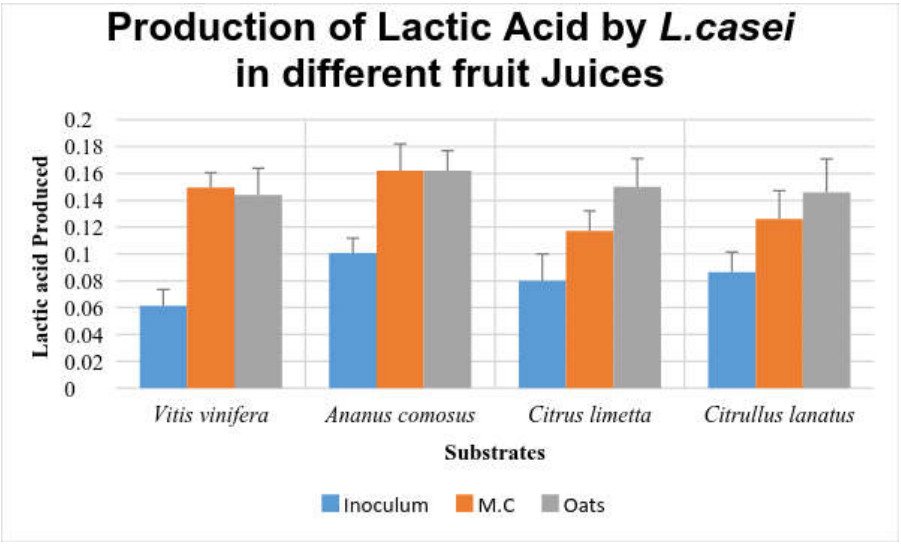

Figure 5. Production of Lactic acid by *L.casei* in different fruit juices

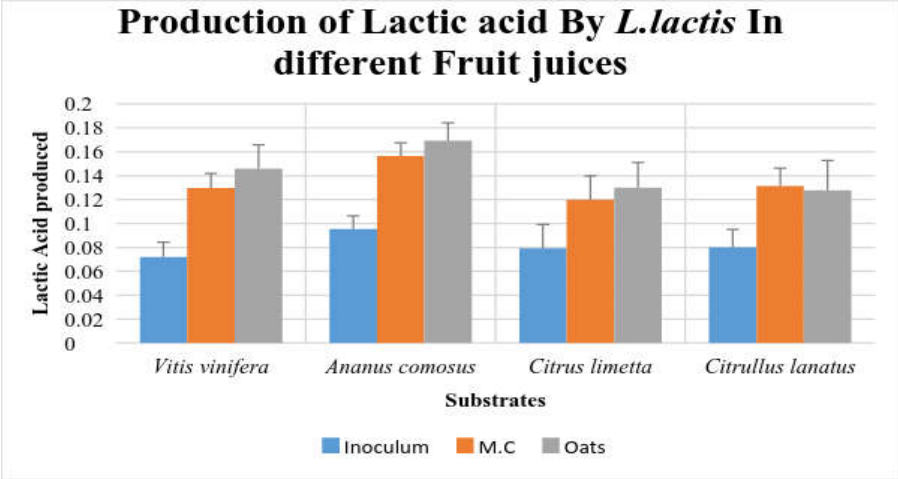

Figure 6. Production of Lactic acid by *L.lactis* in different fruit juices

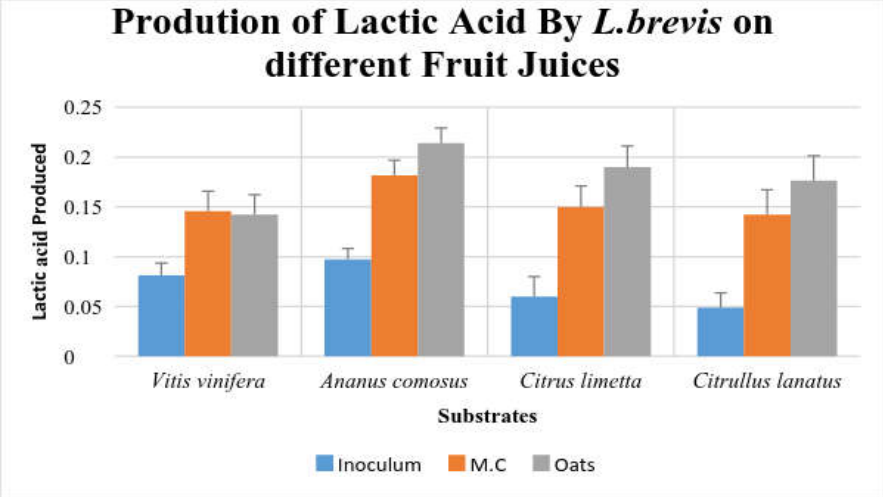

Figure 7. Production of Lactic acid by *L.brevis* in different fruit juices

Growth of LAB strains in the presence of prebiotics

Significant growth was observed in the three different strains of LAB upon addition of prebiotics either singly or in combination. Maximum growth was observed in *L.casei* at 48h and 72h of incubation in the presence of the prebiotic oats in MRS broth. The other strains namely *L brevis and L.lactis* also showed an appreciable growth with the optical density value ranging from 1.377±0.13 to 2.622±0.12 after 48h incubation and 2.1±0.16 to 2.842±0.12 after 72h of incubation. Thus the current study shows that oats in singly as well as combination with pectin can act as a potential prebiotic and can be stable at the gastrointestinal conditions imparting their stabilizing effect on the growth of probiotic bacteria in the human gut. The strains in the figure 1 are depicted as {blue- *L. casei,* light green- *L.lactis,* dark green- *L.brevis*}

Encapsulation and co-microencapsulation

Microencapsulation : 5mL of the cell suspension of the 6 strains were immobilized into alginate beads and were stored in 10mL of MRS broth at 4ºC. The viability of the immobilized beads was found to be higher than free cells.

Co-microencapsulation : 5mL of the synbioticated cell suspension of *L.casei, L.lactis,* and *L.brevis* with oats were immobilized into alginate beads and stored at 4ºC in MRS broth.

Determination of synbiotic potential of fruit juices

Initial Turbidometric Measurements and pH values of substrates

Initial turbidometric measurements and pH values were taken which were later used as controls. Results are tabulated in Table 2.

Turbidometric measurements

Turbidity of the substrates were measured before and after probiotification. All the four fruit juices had shown significant growth of the 3 LAB strains. Studies were done on 6 LAB strains with each fruit juices individually.

Synbiotic Potential of Substrates (Fruit Juices)

In *Vitis vinifera*, it was seen that there was gradual increase in the growth of co-microencapsulated synbiotic product with oats at 72h incubation when compared with other samples. Maximum growth was observed in the synbiotic product with *L.casei, L.lactis* and *L.brevis.* Significant growth was also seen in the microencapsulated gel beads of *L.brevis and L.lactis* at 72h incubation. In *Ananus comosus*, maximum growth was observed in the microencapsulated synbiotic product of *L.casei,* and *L.lactis* after 72hrs of incubation when compared to the other strains. Gradual increase in the growth of co-microencapsulated synbiotic product (with oats) of *L.brevis* was also seen after 72h of incubation. In *Citrus limetta*, appreciable growth was observed in all three LAB strains after 72h of incubation, the maximum growth in the co-microencapsulated synbiotic product (with oats) of *L.casei* and *L.lactis*. The study even showed gradual increase in the growth of the microencapsulated synbiotic product of *L.brevis*. In *Citrullus lanatus,* significant growth was observed in the co-microencapsulated synbiotic product (with oats) of the 2 LAB strains *L.lactis,* and *L.brevis* after 72h of incubation. Maximum growth was even being observed in the microencapsulated synbiotic product of *L.lactis*. Figure 2, 3 and 4 shows the result of synbiotification of fruit juices by different LAB Strains.

Titrable acidity in the Synbiotic Product

Fruit Juices on fermentation produced acids like Lactic acid etc. Hence, acidity of the substrate increased due to probiotification. All the 4 juices showed maximum production of Lactic acid with the Microencapsulated and Co-microencapsulated Synbiotic Product. Production of the acid was minimum with the Inoculum Synbiotic Product. The study showed the strain could better ferment the juices after encapsulation and synbiotification. In *Vitis vinifera,* the microencapsulated and co-microencapsulated gel beads of all three strains equally fermented and produced maximum acid after 72h of incubation. In *Ananus comosus*, the co-microencapsulated gel beads of *L.brevis* strain fermented and produced maximum acid compared to other strains after 72h of incubation. In *Citrus limetta*, the co-microencapsulated gel beads of *L.brevis* and *L.lactis* strains and microencapsulated gel beads of all three strains fermented and produced maximum acid compared to other strains after 72h of incubation. In *Citrullus lanatus*, the co-microencapsulated gel beads of *L.brevis* and *L.casei* strains and the microencapsulated gel beads of all three strains, fermented and produced maximum acid compared to other strains after 72h of incubation. Figures 5,6 and 7 shows the results.

Shelf Life Study

Turbidometric measurements were taken after 10 days till 40 days. Significant drop in the initial pH of the probioticated juices was observed. The viable counts of encapsulated cells were slightly decline, whereas, the viable counts of free cells were remarkably dropped during the storage. The drop in the cell count was because of the production of acid during fermentation. Encapsulation of the cells hence, improved the viability over free cells.

DISCUSSION

Probiotics are defined as viable microorganisms, sufficient amounts of which reach the intestine in an active state and thus exert positive health effects. Prebiotics are non-digestible carbohydrates which acts as food for the survival of Probiotics. Oats and pectin either singly or in combination as a prebiotic has great influence on the growth of LAB strains which are seen in human gut and helps to maintain good health. When probiotics and prebiotics are combined, they form a synbiotics. Fermented products, such as yogurt and different fruit juices, are considered synbiotic because they contain live bacteria and the fuel they need to thrive. The three LAB strains when subjected to the prebiotics (oats, pectin and combination of both) showed maximum growth with Oats and the mixture of prebiotics. *L.casei* showed the maximum growth followed by *L.lactis* after 72h of incubation with oats. With pectin the maximum growth was seen in *L.casei* and in *L.brevis* after 72h of incubation. Upon forming a combination of oats and pectin, the maximum growth was observed in *L.lactis* after 48h of incubation and followed by maximum growth in *L.casei* after 72h of incubation. Hence with the prebiotics either singly or in combination with both *L.casei* showed maximum growth after 72h of incubation. The strains showing maximum growth were subjected to microencapsulation using calcium alginate to form alginate beads. The gel beads were stored in MRS broth at 4ºC for further studies. The encapsulation allows the probiotic strains to survive the gastrointestinal system of the host. Fruit Juices are important sources of saccharides and can serve as a suitable medium for the growth of lactic acid bacteria to enhance the health benefits of the food product. The LAB strains thus encapsulated were subjected to a process of fermentation of four fruit juices (grapes, mosambi, pineapple and watermelon). Fermentation is a traditional way to preserve fruits. Fermented dairy products are the main source of food microbes and dairy products are the most common food matrix for administering probiotics. However, some populations do not consume milk products out of principle or due to lactose intolerance. Therefore, probiotic containing products from plant origin could be a valuable alternative. The four fruit juices fermented with the LAB Strains proved to be potential carrier of the probiotics and formed a better synbiotic product on co-microencapsulation with prebiotics. The fermentation was followed by turbidometric measurements.

It was seen that *Vitis vinefera* was better fermented using all the immobilized strains combined with both the prebiotics(mixture) and showed higher O.D values when compared to other strains. In *Citrus limetta*, the highest turbidity was seen with inoculum of all the LAB strains and immobilized strains of LAB strains with oats. In *Ananus comosus,* the maximum growth of LAB was seen in microencapsulated symbiotic product of all the three LAB strains. The growth rate wasn't much appreciable of *L.casei* as synbiotic product with *Citrullus lanatus* but significant growth was observed in the Co-microencapsulated synbiotic product of the other two LAB strains *L.lactis* and *L.brevis*. The encapsulation of the probiotics in alginate beads can protect the cells inside from the inhibiting compounds like acids and flavonoids in the fruit juices. The viable cell number of immobilized cells was found to be higher than that of free

cells. Immobilized cells which leaked during fermentation grew in the medium as free cells. Some diffusion of cells from the entrapped gel beads occurred during immobilized cell fermentation because of the growth of bacteria in the immobilized gel beads. There are several reports to suggest improved survival rate and efficient activity of the microencapsulated probiotic bacteria. The study showed survival increment of Lactobacillus strains in fruit juices when they were encapsulated within calcium alginate as compared to the free cells. The LAB stains thus survived during the entire storage period of nearly 4 weeks. Several microencapsulated formulations of LAB strains are being tried out with increased efficacy during and after delivery. The production levels and the proportions among these compounds depend on the strain as well as the type of fruits used. The viability of microencapsulated probiotics in fruit juices was seen throughout the storage. Though the fruit juices provided no better environment for the probiotic organism to survive, the probiotics encapsulated in calcium alginate beads survived for up to a month. Although the encapsulation method increases the survival of probiotics in fruit juices, the effect of the encapsulated LAB strains on the sensory characteristic and consumers conception should be analyzed further. The advancements in technologies for fermentation, encapsulation and freeze drying probiotic preparations has proven prophylactic and healing actions. Understanding the synbiotic phenomena between the strains is of prime importance for the control of the cultures and could be used for developing a functionally healthy beverage or functional food to promote health and nutrition of the consumer. Presumptive studies on mechanisms of the probiotic activities may enable their new medical applications for lactose intolerant, diabetic patients and irritable bowel syndrome.

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