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MICROENCAPSULATION OF PREBIOTICS EXTRACTED FROM *CITRUS* SEEDS AND ESTABLISHMENT OF THEIR STABILITY FOR FOOD VEHICLES

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ABSTRACT

Background. Prebiotics provide a survival advantage to probiotics over pathogens. There is a need to identify novel sources of prebiotics that can be introduced into daily diets. In the present study, *Citrus* seeds were identified as a promising source of prebiotics. A combination of probiotics and prebiotics is termed synbiotics; however, crude mixtures fail to last in the gastro-intestinal environment. Microencapsulation is a means of ensuring the stability and effectiveness of synbiotics. When encapsulated synbiotics are introduced into food vehicles, the latter are termed functional foods. The aim of the present paper is to ascertain the shelf-life stability of a functional tomato sauce fortified with encapsulated synbiotics.

Materials and methods. Polar solvents were used to extract the low molecular weight carbohydrates (LMWC) from *Citrus* seeds. The prebiotic activity of the extracts was quantified and they were packed with *Lactobacillus acidophilus* (*L. acidophilus*) (ATCC4356) into microcapsules using emulsion and extrusion techniques. An SEM study was carried out to test the stability and quality of the microcapsules. They were stably integrated into a sauce. The shelf-life of the sauce (pH, acidity, soluble solids, salt content) and release of probiotics from the microcapsules were tested over a period of 50 days. The safety of the food product for consumption was also established.

Results. A positive prebiotic activity score (PAS) of 11.25 was recorded for the LMWC extracted from *Citrus* seeds, confirming their preferential utilization by probiotics. The extrusion beads and emulsion capsules imparted a soft texture to the product. The water activity values of extrusion beads and emulsion capsules were 0.541 and 0.428 respectively, suggesting a lack of contamination by microbial growth. The salt content in the sauce was approximately 1.5% throughout the study. Up to 20 days of storage the acidity decreased and the soluble solids concentration increased. Syneresis was seen to start on the 35th day of storage at cool temperatures, which caused apparent changes in the biochemical parameters of the sauce. *Lactobacilli* were viable even on the 50th day; however, a decrease in their growth was observed.

Conclusion. *Citrus* seed extracts are hitherto unexplored as sources of prebiotics. Two methods of microencapsulation (emulsion and extrusion) were tested to carefully pack the probiotics and prebiotics. The utilization of tomato sauce as a food vehicle was challenging since its low pH had the potential to cause the destabilization of the encapsulation. However, the robustness of the emulsion method was verified, as it yielded a greater CFU/mL of probiotics on release right up to the end of the shelf-life study. Further, the capsules did not affect the textural properties of the sauce in a way that would hinder consumer choice. In the future, this work could be extended with *in vivo* studies to establish the bio-utility of the encapsulated synbiotics.

Keywords: low molecular weight carbohydrates, prebiotic activity score, microencapsulation, *Citrus* seed extracts, tomato sauce

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INTRODUCTION

More than two decades of research on gut biology and the microbiome has shown the significance of probiotics in the health and overall metabolism of any organism (Latif et al., 2003). Probiotics encompass a wide range of bacterial strains including species from the genera of *Lactobacillus* and *Bifidobacterium.* These bacteria have been shown to provide health benefits like improved digestion; alleviation of diarrhea, constipation and symptoms of irritable bowel syndrome; enhanced immune function; improved mental health via the gut-brain axis; immunity boost, etc. Probiotics are naturally found in some fermented foods and beverages, including yogurt, kefir, sauerkraut, kimchi, miso and kombucha (Wenbin et al., 2019). Additionally, probiotic supplements are widely available in various forms, including capsules, tablets, powders and liquid extracts (Yadav et al., 2022). The growth and maintenance of probiotics in the gut requires appropriate nutrients for their growth and protection from drug and alcohol abuse. Prebiotics play a significant role in this process.

Prebiotics are non-digestible fibers that serve as food for probiotics, thereby helping them thrive in the gut. Primarily, prebiotics are certain types of carbohydrates (inulin, fructose oligosaccharides, galactose oligosaccharides, xylose oligosaccharides, polyols, etc.) that are not broken down in the upper gastro-intestinal tract. As a result, they reach the gut and are fermented by probiotics in the microbiome, resulting in beneficial end products including propionic acid, lactic acid and butyric acid. They inhibit the proliferation of harmful bacteria and maintain a balanced microbial community in the digestive system (Davani-Davari et al., 2019). Prebiotics are naturally found in various foods, like garlic, Jerusalem artichoke, bananas, chickpeas and oats (Ishu and Pratyoosh, 2019).

A balanced diet with both prebiotic- and probiotic-rich foods is considered a well-rounded approach to gut health. Combinations of specific probiotic strains and particular prebiotic fractions that promote overall gut health are called 'synbiotics' (Jenkins and Mason, 2022). These are found in various forms, like dietary supplements and functional foods (e.g., yoghurt with specific probiotic strains and prebiotic fibres). Functional foods are designed to provide live beneficial microorganisms while nourishing them with the non-digestible carbohydrates they need to thrive. Such formulations can be tailored to individuals' needs, allowing them to target particular health conditions and populations.

Consumerism is paving the way for the development of these modern super foods, leading to the development of discrete food patterns. A 'prebiotic' is "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson and Roberfroid, 1995). The enzymatic hydrolysis products of *Citrus* peel have been shown to be sources of emerging prebiotics due to their probiotic modulating ability in the gut (Foti et al., 2022). Probiotics bestow many beneficial health effects upon the host, such as butyrate-led induction of apoptosis in cancerous colonocytes (Vadder et al., 2014), generation of hypoxia for maintenance of oxygen balance in the gut (Byndloss et al., 2017), initiation of gluconeogenesis in the liver by propionate and regulation of cholesterol metabolism and lipogenesis by acetate (Frost et al., 2014). In the present study, LMWC were isolated from seeds of *Citrus limon* and evaluated for their prebiotic potentials. The bioavailability of LMWC was increased through encapsulation within materials that are hygroscopic and resistant to gastric digestion. The comparative studies using emulsion and extrusion techniques showed that microencapsulation did not alter the consistency of the food vehicle (tomato sauce, a condiment generally accepted by all age groups). A shelf-life study demonstrated the usefulness of microencapsulation as a safe vehicle for delivering both probiotics (*L. acidophilus*) and prebiotics (LMWC) to the gut.

MATERIALS AND METHODS

All chemicals used in the study were of analytical grade. Ethanol, dinitrosalicylic acid, hydrochloric acid, sulfuric acids and petroleum ether were purchased from Hayman, India. Sodium hydroxide, Potassium hydroxide, silver nitrate, calcium chloride, Glucose, Fructose and Phenol Red were procured from Fischer Scientific, U.S.A. Bacteriological media (MRS – De Man, Rogosa, Sharpe, LB – Luria-Bertani) and agar (MRS, LB)

were purchased from Hi media, India and molecular biology grade chemicals including amylase, pepsin, pancreatin, bile salts, Tween 80, corn starch, sodium alginate and peptone were purchased from SIGMA, India. Completely ripened yellow fruits (*Citrus limon*) were procured locally. *L. acidophilus* (ATCC4356) and *E. coli* (MTCC728) were used in the study.

All experiments were performed in triplicate to ensure consistency.

Establishing the prebiotic activity of CSL

Seeds were washed with distilled water three times and dried in an air oven (Digiqual DQ-OV-03, India) at 80°C until a constant weight (Shimadzu UX420H, India) was achieved, indicating a complete loss of moisture. Dried seeds were crushed into powder and sieved through a 20 µm sieve. Extraction was performed according to the methods described in Wichienchot et al. (2011) with a few modifications. Briefly, 10 g of fine seed powder were weighed, 80% ethanol was added (just enough to cover the sample thoroughly) and the resulting solution was placed in a shaker incubator (Orbitek Scigenics Biotech, India) at room temperature for 24 h. The oil layer was carefully removed and the remaining solution was filtered using a muslin cloth. The denatured proteins were separated from the extract using a 2 µm syringe filter. The ethanol was removed from the filtrate using a rotary evaporator (Equitron Evator, India). The remaining solvent with prebiotic extracts (*Citrus* seed LMWC (CSL)) was lyophilised (Scanvac, India) and stored at 4°C for further use.

The prebiotic potential of the LMWC fraction was evaluated by checking its resistance to gastric and intestinal digestion and preferred utilization by probiotics (Gibson et al., 2010). In brief, the extracted CSL was tested as the sole source of carbon for fermentation by probiotic bacteria in a basal medium without exogenous glucose or beef extract. To check the fermentation product of CSL, 1 ml of 0.5% phenol red was added to a 9 mL medium that contained 0.1 mL of bacterial culture grown overnight. The mixture was incubated at 37°C for 24 h and the appearance of yellow colour from the phenol red was monitored as an indicator of pH change to an acidic range.

The resistance of CSL to gastro-intestinal digestion was analysed as described earlier (Miller, 1959), with minor changes. In brief, 2 g of sample was mixed with 2 mL of pepsin solution (0.576 g pepsin in 12 mL 0.1 M HCl) in a conical flask. The mixture was incubated at 60 rpm and 37°C for 2 h. The flasks were covered with aluminium foil to reduce exposure to light. To this, 5 mL of bile pancreatin (0.12 g pancreatin and 0.5 g bile salts added in 30 mL 0.1 M $NaHCO_3$) and 4 mL of α-amylase (1 mg/mL in Phosphate Buffer Saline) were added and the samples were incubated for 2 h at 60 rpm and 37°C. The digested samples were then centrifuged (REMI, India) at 3000 rpm for 5 min. The supernatant was stored at 4°C. DNS (3,5-Dinitrosalicylic acid) assay was performed on the samples before and after digestion to assess LMWC resistance to pancreatic digestion.

The prebiotic potential of CSL was assessed using a standard protocol (Palframan et al., 2003). In brief, *L. acidophilus* was inoculated in MRS and LB broths in duplicate. *E. coli* was used as a control. Glucose (1% w/v) was added to one set of LB Broth and MRS broth and CSL (1% w/v) was added to the other sets of broth. One percent of each of the cultures of *E. coli* and *L. acidophilus* grown overnight was added to LB Broth and MRS broth respectively. The flasks were incubated in a shaker for 24 h at 37°C. Colony-forming units of bacteria were monitored at 0 h and 24 h of inoculation and the number of colonies was determined using a colony counter (Lapiz, India). The Prebiotic Activity Score (PAS) was calculated as presented in formula (1):

$$PAS = \frac{A}{B} - \frac{C}{D} \quad (1)$$

where

A – probiotic log cfu/mL on the prebiotic at 24 h – probiotic log cfu/mL on the prebiotic at 0 h
B – probiotic log cfu/mL on the glucose at 24 h – probiotic log cfu/mL on the glucose at 0 h
C – enteric log cfu/mL on the prebiotic at 24 h – enteric log cfu/mL on the prebiotic at 0 h
D – enteric log cfu/mL on the glucose at 24 h – enteric log cfu/mL on the glucose at 0 h

A positive PAS value indicates that a carbohydrate is a prebiotic and is better utilized by the probiotic. A negative score indicates that substrate is not a prebiotic since it can be utilized with equal or better efficiency by the pathogenic strains compared to the beneficial bacteria (Baratella et al., 2016).

The prebiotic index (I_{preb}) was calculated as in formula (2),

$$I_{preb} = \frac{\text{CFU of probiotics on prebiotics}}{\text{CFU of probiotics on control carbohydrates}} \quad (2)$$

An I_{preb} of more than 1 indicates strong prebiotic characteristics of the carbohydrate under study and vice versa.

Characterization of encapsulated synbiotics

10 g of corn starch was added to 100 mL of distilled water and the solution was boiled until it formed a gel. 1% sodium alginate was added and the gel was mixed with 2% bacterial cultures and prebiotic powder. The mixture was stirred and suspended in 500 mL of vegetable oil containing 0.2% Tween 80, and stirring continued until it appeared creamy. The mixture was allowed to separate into two phases (oil and water) and then 200 mL of 0.1 M $CaCl_2$ was added. It was kept static at room temperature for 30 min to settle down the encapsulated capsules in the beaker. The oil layer was carefully removed and capsules were harvested by centrifuging at a low speed of 1500 rpm for 10 min. They were stored at 4°C in 0.1% peptone solution for further use.

Extruded beads were prepared by suspending 20 g bacterial cells and prebiotic in 1L of 2% sodium alginate solution. This mixture was added drop by drop using a micropipette into 2% w/v $CaCl_2$ solution under continuous agitation. Once a single layer of gel beads of approximately 3 mm diameter was formed, they were incubated at room temperature in the $CaCl_2$ solution for 1 h to allow the gel to harden. The gel beads were strained through a sterile filter paper and rinsed with sterile physiological peptone solution (0.1 %) to remove the excess calcium ions and free microbial cells. Water activity was measured using a water activity meter (MA35, Sartorius, Germany) as per the manufacturer's instructions. In brief, the sample cup was filled up to 3 mm with the encapsulated beads or emulsion capsules and care was taken to ensure that they did not touch the measurement probe head. The sample cup was placed in a sample holder and the lid was closed. Machine readings were noted in triplicate.

The morphology of the cells was studied using an SEM (Carl Zeiss MA15/EVO18, Germany). A double-sided tape was used to mount the samples on the stub. A sputter coater was used for 6 min to coat the samples. Observations were made using an accelerating voltage of 30 kV with an SE detector.

The cells were tested for their stability in simulated gastro-intestinal conditions following minor modifications to the methods described by Yi et al. (2014). The Simulated Gastric Juice (SGJ) was prepared by dissolving 0.3 g pepsin in 0.5% w/v NaCl and the pH was adjusted to 1.5 with HCl. The Simulated Intestinal Juice (SIJ) was prepared by dissolving 0.1 g pancreatin in 0.5% w/v NaCl with 4.5% bile salts. The pH was adjusted with 0.1% NaOH to 8.0. One gram of encapsulated bacteria was added to 10 mL of SGJ and SIJ and incubated at 37°C for 30, 60, 90 and 120 min. Cell survival was assessed by plating (100 µL) on MRS agar and incubating at 37°C.

The efficiency of the encapsulation was tested in a sterile citrate buffer (pH 6.0) (Zanjani et al., 2014). In brief, one gram of encapsulated cells was added to 9 ml of 1% buffer, kept in a shaker for 15 min and vortexed for 90 sec. For the enumeration of cell release, 100 µL of buffer expected to have cells released from the capsules was plated on MRS agar and incubated at 37°C for 24 h. Encapsulation yield is the number of probiotics cells that survived the process of encapsulation. It was estimated as follows: Encapsulation Yield (EY) = N/No, where N_0 is the number of viable bacterial (CFU/mL) culture and N is the number of viable bacteria (CFU/mL) in microcapsules.

Shelf-life study of the sauce

The tomato sauce was prepared by sautéing and blending 1200 g tomato, 180 g onion, 50 g garlic and 12 g NaCl). The sauce was autoclaved and brought to room temperature. Two batches were prepared with beads (10 g) and capsules (10 g) mixed into the sauce (30 g), and they were stored in glass bottles in a horizontal position at 18°C. The shelf life of 'synbiotic' in tomato sauce was studied for 50 days and samples were analysed for total soluble solids, acidity, salt content and release of entrapped probiotics at regular intervals of 10 days. Since it is difficult to determine salt-free tomato solids and their ingredients in the formulation, considering the salt interference in the quality assessment of the tomato-synbiotics formulation, the concentration of the product was assessed using the

refractive index of the filtrate. Part of the sample was squeezed through muslin cloth and the filtrate was used to determine the refractive index using a refractometer (Atago, Abbe Refractometer, Japan). Data was normalized with respect to variations in temperature as per manufacturer protocol.

$$n^{20}_{D} = n^{t}_{D} + 0.00045\ (t - 20) \quad (3)$$

where

n^{20}_{D} is the refractive index at 20°C

n^{t}_{D} is the refractive index at the temperature of measurement

t is the temperature of measurement, in degrees Celsius.

The salt content was measured according to the standard protocols in the FSSAI (Food Safety and Standards Authority of India) manual of methods for the analysis of foods. In brief, 0.1 N $AgNO_3$ was taken in a burette and 5 to 10 g of probiotic sauce was taken in 100 mL conical flask, and the acidity of the sauce was neutralized with standard 0.1 N NaOH. 1 mL 5% K_2CrO_4 was added and the mixture was titrated against $AgNO_3$ to produce a reddish-brown end point. The percentage salt content was measured with the following formula:

$$\text{NaCl\%} = \frac{\text{Titre Value} \times \text{Normality of } AgNO_3 \times 58.45 \times 100}{\text{Weight of Sample} \times 1000} \quad (4)$$

The acidity of the sauce was determined using a 10-gram sample dissolved in 50 mL of boiled water in a 100 mL conical flask and titrated with 0.1 N NaOH using phenolphthalein as an indicator. The end point was a pale permanent pink colour. The acidity was calculated using the formula:

$$\text{Acidity} = \frac{\text{Equivalent Weight of Acid} \times \text{Normality of NaOH} \times \text{Volume of NaOH} \times 100}{\text{Weight of Sample}} \quad (5)$$

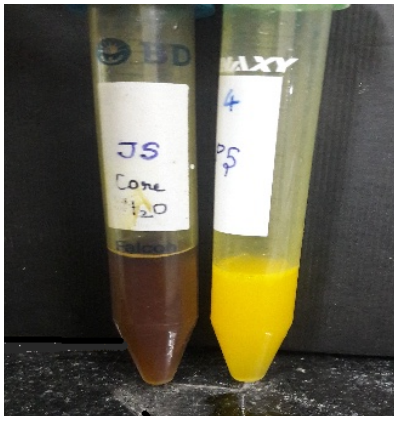
Bacterial growth is given as CFU/mL. All tests were performed in triplicate and the data presented are either representative of a reproducible experiment repeated at least 3 times or consist of means with standard deviations (n = 3). All data were subjected to univariate analysis with Duncan's multiple range test (limited duration free version of XL Stat extension in Microsoft Excel).

RESULTS AND DISCUSSION

Research has brought to the fore that human beings harbour two dominant microbial populations, i.e., *Bacteroidetes* and *Firmicutes*, *a*nd their *r*elative proportions vary on the basis of a person's nutritional intake and health status (Arora et al., 2011). Biochemical pathways to ensure a healthy host gut are only possible when fermentation is affected by microbiota (Falck, 2014).

Establishing the prebiotic activity of CSL

During any fermentation process an organic substrate generally serves as the final electron acceptor with concomitant end-products – acid and/or gas production, generally carbondioxide. The most common acids are acetic acid, formic acid and butyric acid, and they result in increased acidity of the medium and therefore a decrease in pH. In our study, the phenol red broth medium acted as a differential medium by turning yellow in colour to indicate the presence of bacteria that produce acidic end products of fermentation (Fig. 1).



A B

Fig. 1. The assessment of the fermentation of CSL by the probiotic bacterium Lactobacillus acidophilus. The bacterium was inoculated into phenol red broth medium supplemented with CSL as the sole source of carbon. A change in the colour of phenol red to yellow due to a drop in pH upon fermentation of CSL into lactic acid was recorded. A – initial inoculation of bacteria into phenol red broth medium; B – change in colour of the medium after fermentation. The data shown is representative of the experiments, which were repeated at least 3 times.

The prebiotic attribute of LMWC extracted from seeds of *Citrus limon* (CSL) to be used in Ready-To-Eat tomato sauce is reported here for the first time. The amounts of sugar (% w/w) in the CSL before and after the *in vitro* digestion were found to be 46.5 ±2.8 and 38.4 ±1.5 respectively, demonstrating that approximately 80% of the sugars are resistant to digestive action. To measure selective utilization of CSL by probiotics, the I_{preb} was calculated as 2.1, more fully describing the CSL as prebiotic. A positive PAS of 11.25 was computed, which indicated that the substrate under study was a potential prebiotic (Table 1).

Table 1. The growth of *Escherichia coli*, and *L. acidophilus* on glucose and CSL as a sole source of carbon

Substrate	Log CFU/ml Mean ±SD (n = 3)	
	0 hr	24 hr
L. acidophilus (on glucose)	5.579	5.778
L. acidophilus (on CSL)	5.623	6.191
E. coli (on glucose)	5.977	5.944
E. coli (on CSL)	5.88	6.173

L. acidophilus and *E. coli* were grown separately on glucose and CSL broths for a period of 24 h and their growth characteristics were monitored after 24 h on agar plates. The data shown here is the mean ±SD (n = 3).

The log CFU/mL values were used to calculate the Prebiotic Activity Score (PAS) of the extracts. The score takes into account the rate of growth of bacteria rather than the absolute values. This reflects the simple fact that though the carbon sources from prebiotics are equally available to probiotics and pathogens, the rate at which they are metabolized by the individual bacteria is different. The ability of probiotics to exhibit competitive exclusion when exposed to certain carbohydrate fractions in the gut environment is studied in this experiment. Our results indicate that the rate of growth of *L. acidophilus* on prebiotics is higher than that of *E. coli.*

Zarinah et al. (2018) performed similar studies as a quantitative tool to measure the potential of prebiotics in supporting the growth of probiotics. They obtained the highest PAS of 0.45 for *L. plantarum* and 0.65 for *B. bifidum* grown on resistant starch from breadfruit. Their results showcase the fact that a single carbohydrate fraction may have different effects on the growth of varied bacteria. Ribeiro et al. (2020) tested freeze-dried xique-xique cladode juice for its prebiotic potential by measuring its PAS. The authors achieved a positive PAS (0.9 ±0.2–1.1 ±0.3), thereby suggesting that *L. acidophilus* preferred utilization of the juice fraction over glucose as a carbon source. When four different flours from sweet potato roots were evaluated for their prebiotic potential (Albuquerque et al., 2020), a PAS ranging from 0.11 to 0.55 was obtained, demonstrating selective stimulatory effects.

The larger increase in the bacterial cell count of beneficial bacteria like *Lactobacillus* and *Bifidobacteria* in comparison to harmful bacteria like *Clostridium* and *Enterobacteria* on the addition of prebiotics as nutritional supplements indicates the efficiency of the substrate as a potential prebiotic (Mangwani et al., 2012). So all three conditions for a prebiotic – fermentability, resistance to gastric digestion and preferential utilization by probiotics – were demonstrated.

Characterization of the encapsulated synbiotics

A synbiotic preparation of CSL as prebiotic and *L. acidophilus* as probiotic was formulated using microencapsulation. The methods adopted to develop microcapsules are based on our understanding of immobilised cell technologies. The techniques are standardised to suit the physico-chemical requirements of probiotics and prebiotics in practical food applications. Using microencapsulation, droplets of liquids can be packed into stable and viable polymer coatings (Chandramouli et al., 2004). The coatings provide a barrier between the internal milieu of the synbiotic and the external medium of the food product. Protection from the low pH of the sauce, light and moisture, suppression of dispersion and reactivity, and the avoidance of any effect on the sauce flavour profile are all notable advantages of encapsulation (Heidebach et al., 2012). The extrusion and emulsion beads are aqueous hydrocolloids, and the former are larger. The size of the beads formed by extrusion (2–5 mm) depends on size of the needle orifice, the viscosity of the hydrocolloid mixture and the distance between the outlet and the hardening solution; the size of the capsules (<2 mm) produced by emulsification depends on the speed of

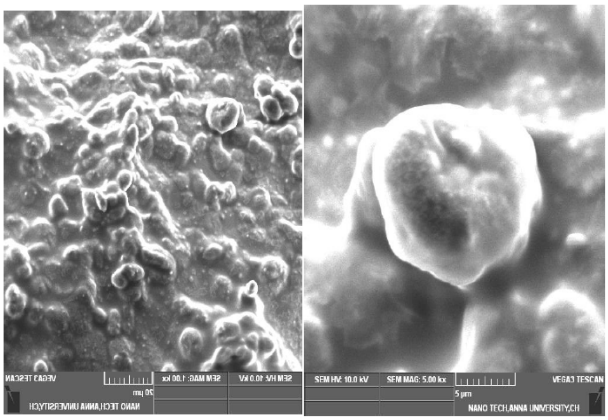


Fig. 2. SEM analysis of prebiotics and probiotics encapsulated in sodium alginate using the emulsion method. The sodium alginate solution containing CSL and probiotic bacterium was suspended in vegetable oil, allowing for phase separation. The lower layer consisting of alginate solution was slowly mixed with calcium chloride and allowed to settle down. Prebiotics and probiotics entrapped in calcium chloride form precipitate in the bottom aqueous layer. The emulsion capsules collected by centrifugation were stored in sterile peptone solution for further use. The figure shown here features the capsules imaged by SEM at resolutions of 1000× and 5000×, respectively. The data shown is representative of the experiments, which were repeated at least 3 times.

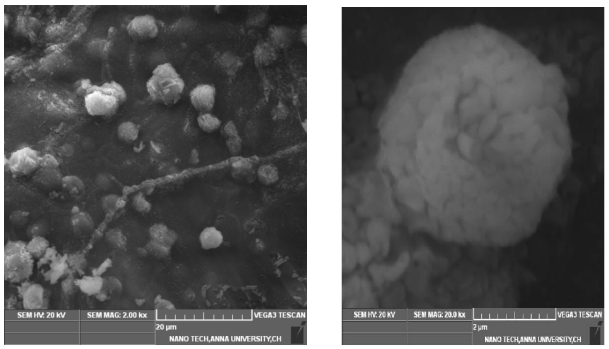


Fig. 3. SEM analysis of prebiotics and probiotics encapsulated in sodium alginate using the extrusion method. 20 g of prebiotics and probiotics were added to a 2% alginate solution and the suspension was added drop-wise into 2% calcium chloride solution under stirring. The beads were allowed to settle down up to 3 mm from the bottom of the beaker, the agitation was stopped and the beads were allowed to harden for an hour. They were separated through a sterile filter paper and stored in sterile peptone solution for further use. SEM images of the capsules were recorded at resolutions of 2000× and 20000×. The data shown here is representative of the experiments, which were repeated at least 3 times

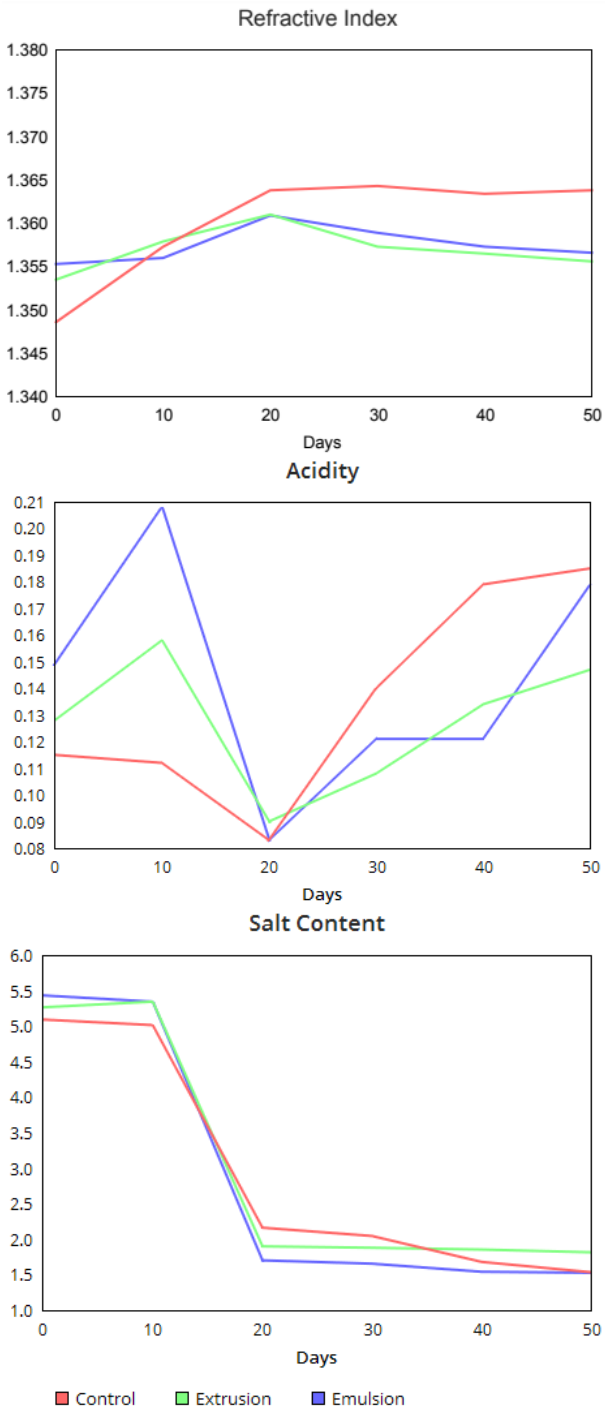
agitation, the type of emulsifier used and the viscosity ratio between dispersed and continuous phase (Kailasapathy, 2002). All microcapsules and beads were found to be spherical in shape (Fig. 2 and Fig. 3).

Microcapsules with a double coating of sodium alginate had a capsule size of 1.89 mm via the extrusion technique (Krasaekoopt et al., 2004). The authors report that the reduction in size of the capsules to less than 100 µm did not effect any significant increase in the probiotic survival rate (Mokarram et al., 2009). The addition of micron-sized probiotics imparts a soft texture to the food product (Cook et al., 2012). In qualitative terms, SEM was used to study the surface roughness of the microcapsules. The emulsion beads exhibited a much smoother texture compared to the extrusion capsules.

The chemical and microbiological stability of the capsules are dependent on their a_w. A low water activity indicates a lower concentration of freely available water that would otherwise allow microbial growth. When the a_w levels are 0.3 and below, it indicates that the water within the product is strongly bound to the polymers, phytochemicals, etc in food and does not play any role in microbiological survival. The water activity values of the extrusion beads (0.541) and emulsion capsules (0.428) were found to be less than the minimum a_w required for the growth of microbes (0.6). The addition of capsules and beads produced either by the method of emulsion or extrusion did not affect the shelf life of the RTE sauce. In a study, the degradation of encapsulated chilli carotenoids was reported when water activity levels reached 0.6 and the dissolution of the biopolymers of microcapsules followed when the a_w levels reached 0.7 to 0.8 (Andrea et al., 2014).

Shelf-life study of the sauce

Tomato sauce was prepared and sterilized by autoclaving at 121°C for 20 min. The standard limit for the incorporation of probiotics into food products should be greater than 108 CFU/g. Based on this limit, 5 g of encapsulated probiotic was added to 30 g of sauce. The sugar content in the synbiotic tomato sauce remained



Samples were measured for refractive indices, levels of citric acid and salts in the formulation at different incubation times. The data shown is the mean of triplicate tests. All the obtained data were subjected to univariate analysis with Duncan's multiple range test (data not shown). Product quality tests were conducted over a period of 50 days at regular intervals of 10 ten days.

Fig. 4. Qualitative analysis of the sauce with microencapsulated synbiotics

the same for a period of 50 days. This shows that the probiotic bacteria incorporated into the sauce did not consume the sugars in the sauce for their growth. Acidity and pH are important parameters in the stability and shelf life of the sauce (Anandsynal et al., 2018). When the pH is low, it inhibits the growth of food-spoiling microbes. The acidity of the sauce during the storage period was between 1 and 3. Acidity in the tomato sauce is due to the presence of citric acid. Salt was added to the tomato sauce to improve the flavour and to preserve the food. Food that contains high salt and sugar tends to spoil slowly. The salt content in the sauce was determined by Mohr's method, which quantifies the NaCl content in the sauce. The salt content in the sauce was 1.5% even after 50 days, and the synbiotic tomato sauce had almost the same salt content as the control tomato sauce (Fig. 4). These results show that the addition of microcapsules to the sauce had no significant effect on the physicochemical properties of the food.

Acid hydrolysis of polysaccharides leading to the formation of mono and disaccharides (sweet in taste) could be the reason for the increase in soluble solids and the corresponding decrease in sourness in the initial stages (up to 20 days). The further increase in acidity could be due to the ionization of neutral molecules like salts, the oxidation of carbohydrates or the degradation of pectin. The onset of syneresis after 35 days of storage could be a contributing factor for the slight decrease in the soluble solid content of the emulsion and extrusion sauces. The increase in soluble solids and acidity could be one reason for the perceived decrease in the salt content after 20 days. Syneresis could also lead to the reduction of the salt concentration in the samples. It could be concluded that the properties of the sauce went through major changes after 20 days of storage. However, these changes were the result of storage at low temperatures, syneresis and the absence of additives and preservatives. Since there was no spoilage of the sauce even at the end of study period, it can be concluded that there was no effect of microbes or microbial activity on the physiochemical properties of the sauce.

Every 10 days, the cell release from the encapsulated beads and capsules into the sauce was determined. The sample was allowed to react with a citrate buffer for 20 min. The buffer liquefies the gel inside the

capsules and/or beads and aids in the release of cells. The results showed that larger numbers of cells were released from the extrusion beads than the emulsion capsules (Table 2).

Table 2. Release of entrapped *L acidophilus* in sauce

Day	Release of cells (log CFU/mL)		
	control	extrusion	emulsion
0	0	5.342	5.255
10	0	5.322	5.136
20	0	5.089	4.982
30	0	5.107	4.986
40	0	5.103	4.968
50	0	5.049	4.934

Synbiotics were encapsulated using the extrusion and emulsion techniques and separately introduced into sauces. The release of bacterial cells in the sauce preparations and their stability were tested. Bacterial counts were monitored for 50 days, as described in methods. The results are shown as mean ±SD (n = 3).

A decrease in the bacterial count was observed in both the test sauces (with extrusion beads and emulsion capsules). The decrease in the number of viable bacterial cells could be attributed to stress due to the microencapsulation and storage of the sauce at a low temperature (4°C) leading to a loss in the viability and functionality of the bacteria.

A challenging product, probiotic bread with encapsulated *L. acidophilus*, was successfully developed by a team using maltodextrin, alginate and xanthan gum. The researchers were able to determine that the survival of the bacteria at higher baking temperatures was attributable to maltodextrin (Thang et al., 2019). In one other similar study, bovine cheese whey was used to encapsulate *L. acidophilus* and its stability was proven in orange juice (Rama et al., 2020). This food vehicle is similar to the one used in the present study in terms of its low pH. It can therefore be understood that microencapsulation can be used as a standard method to deliver probiotics into low pH foods.

CONCLUSIONS

Ongoing research is highlighting the importance of prebiotics to achieve thriving gut microflora. The present study has illustrated that emulsion capsules can hold cells for a longer period of time while also imparting a softer texture to foods in comparison with extrusion beads. It reiterates the applicability of encapsulation as a suitable matrix even in the presence of challenging food matrix conditions like low pH or tough processing conditions like high temperatures. It ascertains the prebiotic efficiency of CSL and the robustness of encapsulation techniques, which ensure its sustainability for a period of 50 days. However, further studies are required to identify multiple stages of synchronized processing that can achieve extension of the shelf-life of food vehicles. There is also a need to perform studies with combinations of probiotics and/or prebiotics to increase the functional properties of the modified food vehicles. Such studies would pave the way for a standardized protocol for the development of many similar functional foods.

DECLARATIONS

Data statement
All data supporting this study has been included in this manuscript.

Ethical Approval
Not applicable.

Competing Interests
The authors declare that they have no conflicts of interest.

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