TECHNICAL FEASIBILITY FOR

COMMERCIALIZATION OF SYNBIOTIC MATRICES

BY

SAIKIRAN CHALUVADI

A Thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Food Science

written under the direction of

Professor Kit L. Yam

and approved by

_____

_____

_____

New Brunswick, New Jersey

January, 2012

ABSTRACT OF THE THESIS

TECHNICAL FEASIBILITY FOR COMMERCIALIZATION OF

SYNBIOTIC MATRICES

by SAIKIRAN CHALUVADI

Thesis Director: Professor Kit. L. Yam

Synbiotics are novel microbial systems that have a high potential in probiotic food applications such as cereal bars, chocolates, jam and jelly based products. Probiotics like *Lactobacillus acidophilus, Lactobacillus reuteri, Bifidobacterium breve,* and *Bifidobacterium longum* are encapsulated by prebiotic fibers such as fructo oligosaccharides, inulin and pectic oligosaccharides to form this synbiotic matrix system.

The role of this matrix is to provide both physical and biochemical protection to the probiotic bacteria during extreme processing and storage conditions enabling their use in a wide range of products. Commercial applications of these matrices require at least 10^7 CFU/ml of probiotic bacteria with an ability to produce short chain fatty acids throughout the product shelf life. Hence, this research focused on a technical feasibility study by measuring the bacteria cell counts from different synbiotic matrices followed by analysis of fatty acids produced during the growth of the same bacteria upon revival from storage, 28 days at 4ºC under aerobic conditions. We were able to retrieve at least 4-logs of bacteria from the synbiotics and they all produced significant amounts (1 to 60 mM) of acetic, butyric, lactic and propionic acids.

Further research was conducted on modifying the synbiotic matrix structure to improve the survival of bacteria. Since the dry pellet form of synbiotic matrices was shown to provide physical protection to the bacteria from storage conditions, the physical form of

the matrix should be changed to hold more moisture to utilize the biochemical properties of these prebiotics. By eliminating the calcium chloride cross-linking step in the matrix preparation protocol to obtain a gel like matrix structure, we achieved an improved survival of bacteria to a minimum of 7-logs throughout the storage period. We also found no effect of relative humidity on the survival of these bacteria when stored in gel based synbiotic matrices. These benefits will help in utilizing these matrices in multitude of food applications provided further research is done on optimizing their structural stability. Overall, synbiotics have proven to be an effective way of protecting bacteria and also providing prebiotic fiber at the same time to the host.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my advisor, Professor Kit L. Yam, for his patience, support and guidance throughout this study. It has been a great experience working under his guidance.

I would also like to thank my committee members Dr. Arland Hotchkiss for his valuable guidance throughout this project and Dr. Linshu Liu for his useful suggestions related to development and testing of matrices. I would also like to thank both these members for taking time to serve on my committee.

Special thanks to all my lab mates for their assistance, discussion, friendship throughout my stay at Rutgers University.

I would also like to thank Dr. John Phillips, Dr. John Luchansky, Doug Soroka, Dr. Vijay Juneja from ERRC, USDA for their suggestions, technical assistance and resources. Special thanks to Angie Martinez, Jeffery Call and Andre White of USDA for helping me out with my lab experiments. I am very grateful to USDA-ARS for providing the funding and required resources for this project.

Finally, special thanks to my family members and friends for their constant support and encouragement.

Table of Contents

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1. Probiotics

1.1.1. Definition of Probiotics

The human gastrointestinal tract is made up of complex consortia of micro-organisms (more than 400 bacterial species) that interact with the host. They include harmful bacteria such as various gram positive cocci, enterobacteria and *E. coli* that may have an adverse effect on the digestive health [1]. Bacteria such as *Enterobacteriacea*, *Enterococcus* and *E. coli* are known to produce harmful carcinogens and toxins, putrefy intestines, and result in diarrhea, constipation and a number of intestinal disorders. Probiotics can be defined as beneficial microbes, predominantly belonging to the genera of *Lactobacillus* and *Bifidobacterium*, fights pathogenic bacteria and promotes the health of an individual. Over the lifetime of an individual these organisms are introduced through various sources, especially milk and milk products. A good probiotic organism exhibits characteristics such as i) non-pathogenic and non-toxic nature, ii) ability to survive and metabolize in gut, iii) retains viability during storage and use and iv) should have good sensory properties when incorporated in food. These organisms, are facultative anaerobes and acid producers, have a major role in reducing the incidents of chronic intestinal inflammation, diarrhea, constipation, irritable bowel syndrome, sepsis, food allergies and liver disease [2]. The primary role of these bacteria is to proliferate inside the lower intestines and establish a homeostatic environment. This environment leads to multiple useful interactions between the host and the probiotics. Microbe-intestine interactions have shown to promote the immune function by enhancing production of

antibodies from dendritic cells and they are capable of exerting an antimicrobial action using various mechanisms.

1.1.2. Mechanism of action

Probiotics have an intestinal barrier function, which is an indirect defense mechanism, achieved by competing with the pathogens for intestinal attachment sites and growth nutrients. This prevents the adherence, proliferation and invasion of pathogenic bacteria. Systemic immune functions are also achieved by allowing the intestinal epithelial cells to produce bioactive factors which are responsible for apoptosis (death) function that helps in preventing colon cancer and other intestinal disorders [2,3].

Probiotic microorganisms also exhibit direct defense mechanisms by fermenting undigested polysaccharides inside the gut and producing compounds that i) have an antimicrobial effect, example, short chain fatty acids (SCFAs) like lactic, acetic, propionic and butyric acids that lower the pH of the gut, ii) modifies the gene expression in resident microbes and reduces their potential to grow and multiply, iii) alter lipid metabolism in host, lower plasma lipoprotein levels and stimulates glycolysis [2].

Different probiotic strains exhibit different intestine adhesion patterns, acid and bioactive compound producing abilities. Multiple levels of action provide a holistic effect on the amount of pathogenic bacteria that resides inside the gut. Therefore no single mechanism can be completely responsible for the overall systemic health benefits provided by probiotics [3]. However, the action of short chain fatty acids on pathogens is well established and easy to analyze. It is also convenient to replicate these mechanisms in

most of *in vitro* and *in vivo* systems that involves probiotic organisms. Hence in this research, we studied the SCFAs production ability of various probiotic strains.

1.1.3. Criteria for probiotics

Apart from the characteristics that were discussed earlier, a probiotic organism should also meet few important criteria for a commercial application:

a. It is needless to mention that the probiotic organisms should be alive throughout the shelf life of a product. However, this distinction has to be made because even dead probiotic organisms were found to trigger a beneficial immunological effect in the host [19].

b. The dose needed for an intended health benefit should be pre-defined based on the strain type and the product. *Bifidobacterium infantis* reduced the symptoms of inflammatory bowel syndrome when 8 logs CFU/ml [4] was used, where as a pharmaceutical product, VSL-3 required more than 10 logs CFU/ml of a probiotic cocktail to reduce the symptoms of diarrhea. However, traditionally 7-8 logs of bacteria are the minimum amount required for any health benefit [10, 28].

c. From the mechanisms that were discussed earlier, probiotic organisms compete with the harmful bacteria for intestinal adhesion sites. Organisms should also produce short chain fatty acids (SCFAs) to reduce the pH of the surroundings and kill harmful bacteria like *E. coli* and other cocci. Hence producing SCFAs even after the end of shelf life of product is another important criterion.

1.1.4. Probiotic products

Active probiotic cultures are most commonly delivered in dairy products and probiotic fortified foods. Freeze dried organisms are also incorporated in dietary supplements and nutraceutical product forms like tablets and capsules [4]. Cholesterol and lactose intolerance are two factors preventing consumption of dairy products on a regular basis, creating a setback to traditional dairy related probiotic products [5]. Fruits and vegetable juices, cereal and meat products are excellent non-dairy substrates for survival and growth of probiotics. They provide excellent pH and other storage conditions. *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei* survived at levels greater than 7.0 log CFU/ml in orange juice and above 6.0 log CFU/ml in pineapple juice for at least 12 weeks [6]. *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* were both found to ferment and survive in tomato, cabbage juices at levels greater than 7 log CFU/ml for 4 weeks at 4ºC [7,8]. Whole grains are excellent sources of carbohydrates (soluble/insoluble), proteins, vitamins, and minerals, oligosaccharides (fructo- and galacto-) that can simulate the growth of complex nutrient requiring lactic acid bacteria (LAB) [9, 10]. Grains such as maize, barley, oats, soybean, rice, and wheat due to their chemical composition can support the growth of organisms such as *Lb. reuteri*, *Lb. acidophilus* and *Bifidobacterium bifidum, Lb. plantarum, Lb. rhamnosus and Lb. fermentum.* Ancient grain based fermented products like Tarhana, Kishk, Ogi, Boza etc. have shown growth, survival and the fermentation abilities of some above mentioned strains [11-16]. Oat based beverages have shown high viability of probiotic organisms after 21 days of refrigerated storage. *Lb. pentosus* and *Lb. plantarum* have been

traditionally used to ferment some Scandinavian type meat sausages also exhibit probiotic properties [17].

1.1.5. Drawbacks of probiotic products

Probiotics can be incorporated into a variety of food products but there are some drawbacks associated with each of these applications.

i. Unsuitable aromas, flavors have been reported with various strains of lactobacillus especially *Lactobacillus plantarum* in fruit juices [18]

ii. Different microorganisms have different sensitivities towards pH of substrate, temperature of surroundings, post acidification in fermented products [8] and overall gastrointestinal conditions. Hence the stability of these probiotic bacteria is not consistent.

iii. Processing conditions like high temperature and pressure required for pasteurization process has shown an adverse affect on the final counts of organisms like *Lactobacillus acidophilus, Lactobacillus rhamnosus etc.* in the dairy and non-dairy beverages [19]

iv. Bifidobacterium strains are less acid tolerant and more oxygen sensitive than Lactobacillus strains. However the counts of both these organisms are reduced during the shelf life of fermented food products [20]

v. Freeze/ Spray drying were found to be the reasons for lowered probiotic counts in many food products [21-24]

Currently, multiple approaches are being investigated to protect the bacteria during processing and storage of probiotic food products. Encapsulation of various strains of

lactobacillus and bifidobacterium in sugars, insoluble dietary fibers like alginates, starches, and whey proteins have given them physical protection during drying, freezing and high temperature processing [25-27]. Immobilization of bacteria on agar, calcium pectinates and alginates has given protection from post acidification after fermentation of products [19].

1.2. Prebiotics

1.2.1. Definition

Prebiotics are dietary fiber components currently defined as "selectively fermented ingredients that allows specific changes, both in the composition and/or activity of the gastrointestinal microbiota that confer benefit(s) upon host wellbeing and health" [29]. These prebiotics are non-viable entities that selectively simulate the growth of probiotic organisms especially Lactobacilli and Bifidobacterium species. However studies have shown prebiotics to be more specific towards Bifidobacterium strains. Some of the prebiotics that were proven effective towards increasing the growth of probiotics were lactulose, inulin, inulin type fructans like fructooligosaccharides and trans-galactooligosaccharides [29]. There are other potential prebiotic fibers like xylooligosaccharides, isomaltooligosaccharides and pectic oligosaccharides (POS). Animal studies and few human trials showed number of benefits associated with some of the above mentioned prebiotics that include managing ulcerative colitis, controlling varieties of diarrhea, and improving calcium absorption [29].

Existence of probiotic bacteria inside the gut is one of the major assumptions of using prebiotic fibers. However, it can be more beneficial to the host if both prebiotics and

probiotics are administered at the same time which can be done by encapsulating the probiotic bacteria within prebiotic fibers.

1.2.2. Prebiotics in food applications

Currently prebiotic fibers are used as nutritional supplements and are part of many functional foods. They are used in food formulations for both organoleptic and nutritional advantages. Infant formula, soups, sauces, confectionary foods, chocolates, cakes, biscuits, meat products, fillings, beverages, yogurts and desserts are some of the product categories in which prebiotics are currently used [30]. Some of the functional properties of prebiotics are fat or sugar replacement, improved texture and mouth feel, fiber, foam stabilization, stability, moisture retention and heat resistance. Prebiotics not only help the growth of probiotic organisms but also has a positive effect on short chain fatty acids (SCFA) production inside the gut. Acids like acetate, propionate, butyrate and lactates supplies additional energy to the host and also acidifies the colon to prevent the growth of pathogenic bacteria [30]. These properties collectively give prebiotics myriad of potential applications in food.

1.3. Synbiotics

1.3.1. Definition of Synbiotics

A synergy between probiotics and prebiotics is termed as a synbiotic system. This can be a simple mixture [31, 32] or a micro-encapsulated form [33] of probiotics aimed at maximizing the benefits of both entities by providing either an additive or synergistic effect. The primary intention of using a synbiotic is to give a layer of protection for the bacteria during their travel through the gastrointestinal track [34]. Alginate is a matrix

polysaccharide used to micro-encapsulate probiotic bacteria and improve their gastrointestinal viability [33, 35]. Our previous study showed that a calcium alginate synbiotic can also have a beneficial effect on the survival/viability of probiotic bacteria during refrigerated storage conditions [36] and this could be an alternative to preservation techniques such as freeze/spray drying.

1.3.2. Literature review

Over the past decade large amounts of research has been done in the area of synbiotics that includes developing and testing various systems, evaluating their *in vitro* and *in vivo* performance, antimicrobial activities, stability studies and health benefits.

Table 1: Functional properties of synbiotic systems

Synbiotic System	Objective	Results
1) Glucooligosaccharides + 5 strains each of Bifidobacterium and Lactobacillus in growth media [37]	1. Resistance to gut fluids 2. Growth inhibition of pathogenic bacteria	1. All Bifidobacteria except *B. longum* and all Lactobacilli except *L. acidophilus* and *L. buchneri* showed higher resistance in both gastric and intestinal juices 2. Higher inhibitory activity by *Bifidobacterium breve* against Gram+ bacteria (*C. difficle & E. faecalis*) whereas *Lactobacillus farciminis* inhibited both G+ and G- bacteria (*E. coli, L. monocytogenes, S. typhi*)
2) Oligofructose/ Fructo oligosaccharides/ Inulin + 9 strains of Bifidobacterium	1. Effect of prebiotic on growth of probiotics	1. 1.1-5 folds higher growth of probiotics in presence of prebiotics

	Objective	Results
in minimal media[38]	2. Probiotic and pathogen counts inside rat intestines after 14 days of simultaneous administration of probiotics and prebiotics	and FOS had higher growth rates 2. 0.6-1.6 logs higher probiotics in faeces compared to control group without prebiotics (significantly high) 3. No effect on overall coliform count but aerobic and anaerobic spore counts restricted to 2 logs.
3) Neosugar (glucose, fructose, oligofructose) + *Bif. breve, Bif. longum, Bif. animalis* in semi solid media [39]	1. Evaluate bile salt resistance in presence of prebiotics	1. Increased bile resistance in presence of oligofructose when compared to glucose and fructose alone
4) Soy germ powder (oligosaccharides) + *Lactobacilli reuteri* in minimal media [40]	1. Survival of bacteria in bile salts in presence of soy powder 2. Fermentation of β-glycosidic isoflavones by bacteria	1. Improved survival in presence of soy germ powder 2. Production of aglycone isoflavone by fermentation
5) Fructo oligosaccharide + *Lactobacillus acidophilus* in skim milk media [41]	1. Evaluate the effects of synbiotic product in artificial gut system 2. SCFA measurement and analysis	1. Higher amounts of lactobacillus (0.89 logs) in ascending colon and higher amounts of bifidobacterium throughout the colon due to prebiotic 2. Inhibition of E. coli, enterobacteria growth 3. Increased butyrate production (3-10 times), acetate and propionates (1-5times)

Table 2: Preservation of probiotic bacteria

Encapsulation System	Objective	Results

1) Entrapment technique: Pectic oligosaccharide/ Fructo oligosaccharide crosslinked with alginate + *Lactobacillus acidophilus/ Lactobacillus reuteri* [36]	1. Study survival of probiotic bacteria within synbiotic matrices	1. Bacteria viable in the matrices after 30 days of refrigerated storage 2. Analysis was qualitatively determined by turbidity changes and scanning electron microscope analysis
2) Syringe extrusion technique: Starch/Alginate + *Lactobacillus acidophilus* for yogurt biomass application [42]	1. Evaluate the viability of cells based on lactic acid production rate in milk	1. Fermentation of milk with respect to acid production was consistent with non encapsulated bacteria 2. System better than chitosan encapsulation due to inert nature of alginate matrix towards bacteria
3) Emulsion technique: 3% Alginate + vegetable oil for frozen ice milk application [43]	1. Evaluate the viability under frozen milk conditions	1. 90% cells survived due to encapsulation as against 40% cell survival in non-encapsulated form after freezing
4) Spray drying: Strains of *Bifidobacterium longum* + glycerol/skim milk/starch/gum Arabic [44]	1. Study effect of spray drying in different media on survival of bacteria 2. Identify the best media that gives maximum protection	1. 2-3 logs reduction in absence of carrier media regardless of strain type. 2. *Bifidobacterium longum* was least sensitive to spray drying in presence of skim milk. 3. Different %s of media had varied effect on survival of specific strain.
5) Sugars + lactic acid bacteria [45]	1. Elucidate mechanisms of protection offered by sugars during various processing steps and storage.	1. Osmotic regulation during washing, drying and storage. 2. Alteration of fermentation metabolites 3. Membrane phase transition during drying, keeping the lipid bilayer intact 4. Cryoprotection during freezing by preferential exclusion of microbes. 5. Prevents excessive water loss during thermal processing.

		6. Increases Tg of starter culture hence reduces the molecular mobility and reactions inside the cells. Hence increases survival under various storage conditions. 7. Protective effect of trehalose>maltose>sucrose>glucose as Tg values increase from glucose to trehalose.

Based on the literature review, following conclusions can be made about the current research in encapsulated probiotics:

1. Probiotic bacteria are sensitive to extreme processing, storage and environmental conditions.

2. Encapsulation offers protection to the bacteria

   a. During their travel down the gastro-intestinal cavity and in contact with bile salts and juices.

   b. During processing like drying, thermal treatment and freezing.

   c. During storage at various environmental conditions like high/low water activity, temperatures, oxygen concentrations, metabolites, pH etc.

3. Synbiotic systems have a beneficial effect on probiotics and some of the synergistic effects include

   a. Higher acid and bile resistance.

   b. Higher growth of host beneficial microflora.

   c. Increased short chain fatty acid production.

   d. Increased reduction of harmful bacteria inside gut/intestines.

4. Physical protection will be offered by many systems like alginates, starches, gelatins, sugars and prebiotics. However, selection of an encapsulation system depends on multiple factors like application type (food/biomass for production/animal feed etc), cost, nutritional qualities, bacteria strain type and processing/storage conditions. Hence a universal encapsulation system is highly impractical.

5. In some cases, Bifidobacterium species survived better than Lactobacillus in spite of the former one being more sensitive to environmental conditions. However, the trends of survival are very inconsistent and no definitive conclusion can be drawn.

Table 3: Application of encapsulated probiotics in foods [46]

Encapsulation System	Food Application
Calcium Alginate + *L. bulgaris/S. thermophilus*	Capsules/ food supplement
Carrageenan + *B. bifidum*	Cheddar Cheese
Skim milk + *L. paracasei*	Cheese
Alginate + *L. acidophilus/ B. bifidum*	Kasar
Alginate/pectin + *L. casei*	Yogurt
Raftilose/Raftiline/Starch + *L. acidophilus/ B. infantis*	Yogurt desserts
Calcium Alginate + *L. lactis*	Cream
Alginate + *B. bifidum/ B. infantis*	Mayonnaise
Starch + *B. PL1*	Dry beverage
Calcium alginate + *L. reuteri/ B. longum*	Sausages
Whey protein + *L. rhamnosus*	Biscuits

Oils/*starch* + *L. salivarius, B. longum, L. plantarum, L. acidophilus, L. paracasei, B. lactis*	Fruit/ vegetable juices
Fatty acids + *L. helveticus/ B. longum*	Chocolates

These are various products that have different encapsulated probiotic systems and it was found that a combination of Bifidobacterium/ Lactobacillus species is predominantly encapsulated with alginate/starch.

1.3.3. Research gaps and opportunities

Based on the literature review conducted the following gaps and opportunities are identified:

1. Most of the studies conducted in this field have focused on improving the survival of probiotic bacteria by encapsulating in non-prebiotic matrices/formulations. It would be very beneficial to use prebiotics in the encapsulation system due to a possible combined biochemical and physical interaction with the bacteria during processing, storage and consumption.

2. Not many studies are available that shows the effect of encapsulation on the growth characteristics of probiotic bacteria. This is important because the bacteria should exhibit same/better growth characteristics than starter cultures.

3. It is important to test the fitness of probiotic bacteria after subjecting them to extreme processing or storage conditions. They are no longer regarded as probiotic if short chain fatty acids are not produced during their growth. None of the studies related to

encapsulated bacteria storage have performed SCFA analysis on revival of bacteria from storage.

4. From earlier research study of Hotchkiss et.al, [36] it was found that the prebiotic matrices kept the bacteria viable at aerobic refrigeration conditions for 30 days. The study was qualitative in which the viability was determined by the change in turbidity of growth media followed by confirmation with scanning electron microscopic analysis. The analysis showed the internal and external structure of the matrix, bacteria predominantly surviving inside the matrix. This study definitely showed the positive effect of using a prebiotic but there is a need to quantify the protective effect and differentiate it from alginate.

5. In order to commercialize the synbiotic matrices there is a need to test previously discussed criteria for probiotic bacteria after storing these matrices for a specific amount of time at specific environmental conditions.

1.4. Objectives

1.4.1. Overall Objective

In order to address the research gaps, we devised objectives to develop and test the synbiotic matrices. The overall objective was to create various combinations of synbiotic matrices and perform a technical feasibility study for commercializing them. If there is a need, the survivability of bacteria within these matrices should be improved.

1.4.2. Scope of the research

Encapsulation of probiotic bacteria, both within prebiotic and non-prebiotic fibers or formulations showed a very positive effect on the overall survival of bacteria. Even in the

previous synbiotic matrices research [36], *Lactobacillus acidophilus* and *Lactobacillus reuteri* survived both in prebiotic (inulin, pectic oligosaccharides, and modified citrus pectin) and also in non-prebiotic (alginate) fibers. In this study, the focus was on the same system but also including Bifidobacterium species. Aerobic refrigeration conditions (approximately 4ºC) were used to mimic the standard storage temperature for most of the existing probiotics products (beverages, meat, yoghurt, jams, milk products like cheeses). Quantities of bacteria, growth characteristics and SCFA production abilities have not been previously evaluated for the current synbiotic system. The protective effect of synbiotic matrices was evaluated only during storage but not processing. Hence the research would focus on these entities along with an exploratory work in the area of improving survivability, deducing preliminary mechanisms for observations and extrapolating findings to future work.

1.4.3. Specific Objectives

Objective 1 - Technical Feasibility Study: Quantitative Analysis

Sub-objectives

1. Measure survival of probiotic strains stored within synbiotic matrices at 4ºC for 4 weeks.

2. Analyze short chain fatty acids produced during growth of stored probiotics upon revival.

3. Identify best prebiotic fiber by analyzing growth characteristics of probiotic organisms.

4. Scanning electron microscope analysis of bacteria stored within these matrices after 4 months of refrigerated storage.

Objective 2 – Improve survival

Sub-objectives

1. Improve the survivability of probiotic bacteria within synbiotic matrices by modifying the physical form of matrix.

2. Study the effect of relative humidity on the survival of bacteria within these matrices.

3. Elucidate a possible mechanism for the effect of environmental factors that influence the survival of bacteria.

1.4.4. Challenges

Biological systems have too many variables that cannot be accounted for during the design of experiments. Their survival primarily depends on the surrounding temperature, pH, oxygen and moisture content but secondary factors like metabolites produced during stress could alter their composition and viability. Hence obtaining an accurate count for each sample is a challenge, which was addressed by taking a larger sample size to account for all extraneous factors. Also preparing HPLC samples for organic acid analysis involves a centrifugation process to separate the cells from media during which volatile acids will be substantially lost. Due to limitation in the experimental apparatus setup a larger sample size was used. The growth characteristics were analyzed with a novel scoring method which might needs to be further scrutinized.

1.4.5. Experimental Variables

1. Composition variables-

- Bacteria strain type and fiber type are the major variables that affect the final cell count after the storage time

- The moisture content of the matrix will have an effect on the survival of bacteria

2. Environmental variables-

   - The bacteria should be incubated at optimum temperature and anaerobic conditions for their growth

   - The matrices should be stored in commercial product storage conditions

2. MATERIALS AND METHODS

2.1. Description of materials

2.1.1. Prebiotics

Three types of prebiotics (oligosaccharides and polysaccharide) here on referred to as POS, FOS, and I along with a non-prebiotic fiber alginate (A) that provides structure to the synbiotic matrix were used in this research.

Table 4: Fibers

Fiber Common Name	Commercial Name	Company
Pectic-oligosaccharides (POS)	POS-II	EcoNugenics
Fructo-oligosaccharides (FOS)	Raftilose P95	Orafti
Inulin (I)	Raftilose Synergy 1	Orafti
Alginic acid (A)	Sodium salt, Type IV	Sigma

- POS II is derived from pectin, which is a polysaccharide consisting mainly of a homogalacturonan backbone that is partially methyl esterified. Homogalacturonan is interrupted periodically by regions of alternating D-galacturonic acid and L-rhamnose residues. Commercial pectin consists of 90% homogalacturonan and 10% rhamnogalacturonan. POS was produced by enzymatic degradation in a continuous ultra-filtration membrane reactor. POS II has a bimodal distribution with 3.8 kDa and 0.97 kDa average molecular weight values along with 2% degree of esterification. It has a degree of polymerization of 4 and has both rhamnogalacturonan oligosaccharides and oligogalacturonic acids [47]. Like other dietary fibers, pectin

reaches large intestine intact and breaks down into POS and other metabolites by enzymatic degradation.

- FOS is a mixture of oligosaccharides which are composed of fructose units connected by β-(2→1) links. Some of the molecules are terminated by a glucose unit. The degree of polymerization (n) generally varies between 2 and 8 [48, 49]. These molecules are known to exhibit prebiotic properties i.e., non-digestible fibers that reach the intestines intact and exhibits strong bifidogenic properties.
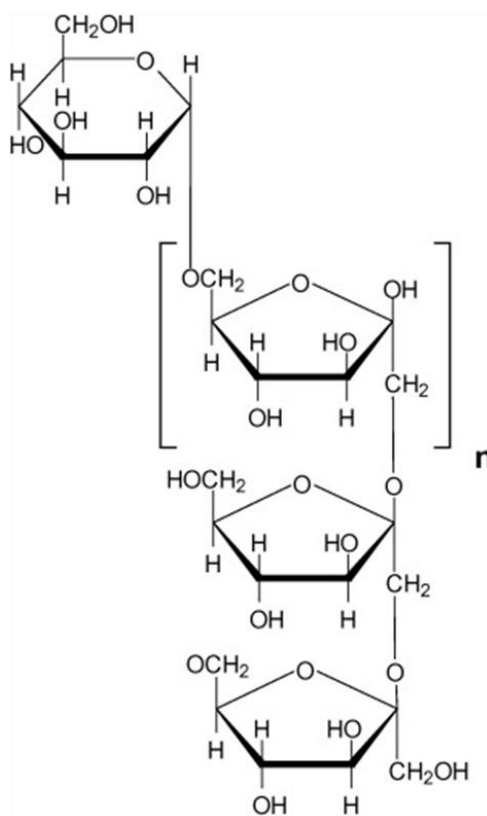
Figure 1: Structure of Inulin type fructo-oligosaccharide

- Inulin is a polysaccharide belonging to the class of fructans. The monomeric D-fructofuranose units are linked by β-(2→1) linkages as shown in figure above. The degree of polymerization generally varies between 2 to 60. It is currently used as a

low calorie fat substitute and non-digestible dietary fiber that exhibits prebiotic properties [50].

- Alginate is a family of unbranched binary copolymers with varied composition and sequence of (1→4) linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues whose sequence and structure depends on the source organism. We used a sodium salt of alginic acid which has a tendency to form highly viscous aqueous solutions and also form stable gels when Na^+ is replaced by Ca^{2+} ion [50]. There are multiple applications of alginate and it gives strength and support to the synbiotic matrix. It is not a prebiotic but was extensively used in earlier studies to protect probiotic bacteria.

Figure 2: Alginate Structure with M and G units
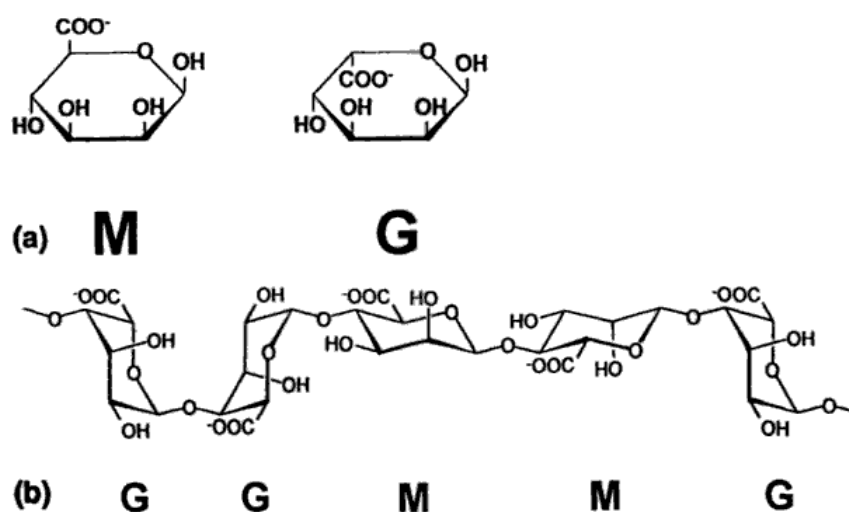
2.1.2. Probiotics

Table 5: Probiotic Organisms

Organism Name	Identification Number
Lactobacillus acidophilus	Luchansky 1426
Lactobacillus reuteri	Luchansky 1428
Bifidobacterium breve	2141, ATCC 15698
Bifidobacterium longum	3300, ATCC 202078

We chose both Lactobacillus and Bifidobacterium species for our study as they are most commonly studied probiotic bacteria types. Lactobacillus species were borrowed from the culture collection of USDA-ARS, ERRC and Bifidobacterium species were taken from ATCC collection. The effect of storage conditions will have a varied effect on both these species due to differences in tolerance to stress conditions.

2.1.3. Reagents

Table 6: List of chemicals

Chemical	Function
Deionized water	For preparing aqueous solutions of fibers, growth media, sterile water for serial dilutions etc.
CaCl$_2$ Solution	Ca^{2+} binds alginate chains together and also crosslink alginate with other fibers
MRS Broth, Difco	Growth media for lactic acid bacteria with pH: 5.5-6.0
BHI Broth, Difco	Minimal media for reviving bacteria from storage with pH: 7.0 mimicking intestines

Sulphuric Acid	Eluent for HPLC
Acetic acid, Butyric acid, Lactic acid and Propionic acid	Standards for short chain fatty acids for HPLC

2.2. Methods

2.2.1. Synbiotic Matrix Preparation

A solution of high viscosity alginate (A; 10 mg/ml) was prepared in deionized water with each of the other oligosaccharides and polysaccharides (POS, FOS, I; 10 mg/ml). A solution of alginate (A; 10 mg/ml) alone was also prepared. The resulting solutions (POSA, FOSA, IA and Alginate) were pipetted into a 96-well titer plate (120 uL/well) and placed inside a freezer (-20ºC) for 45-60 min, followed by lyophilization. A 45 mM calcium chloride solution was added to each well for at least 60 minutes. These matrices were then washed with deionized water in a beaker (3x). The calcium cross-linked matrix plug was then returned to a 96 well plate, placed in a freezer (-20ºC) and lyophilized again [36]. The effect of calcium on the survival of bacteria was studied by repeating the above procedure without adding the calcium chloride solution.

2.2.2. Preparation of cultures

Probiotic bacteria were grown in deMan, Rogosa and Sharpe (MRS) broth (pH 5.5-6.0, Difco) [51] in an anaerobic chamber (5% H_2, 10% CO_2, 85% N_2, 37ºC; Bactron IV, Shel Lab). Each matrix plug within the 96-well plate was inoculated with 15 µl of bacterial cultures (10^8-10^9 CFU/ml) in replicates. Four 96 well plates were stored at 4ºC for 4

weeks under aerobic conditions. Two additional 96 well plates were stored at 4ºC for 4 months under aerobic conditions for SEM analysis.

2.2.3. Survival study for calcium cross linked matrices

A plate was removed from the refrigerator after every week and matrix plugs were inoculated in the glass bottle containing 200 ml of bovine heart infusion (BHI, pH 7; Difco) broth. The bottles were shaken until the matrix completely dissolved and incubated at 37ºC in an anaerobic chamber. At specific time intervals (t = 0, 4, 10, 24, 48 h), 1 ml of broth was pipetted out of the incubated bottles, serially diluted and spread on MRS (Difco) agar plates. Another 1 ml of the broth was frozen in a plastic tube (Eppendorf) for short chain fatty acid analysis. The samples were plated in quadruplicates and incubated inside the anaerobic chamber for 24-48 hours followed by plate counting. Bacteria stored in alginate matrix are the non-prebiotic control in these experiments. Differences in 0 h plate counts after 7, 14, 21, 28 days of storage at 4ºC was checked for significance using a 2-tailed t-test with unequal variance. This was done to evaluate the number of viable cells over the storage period.

2.2.4. Survival study for non-calcium cross linked matrices

The procedure for evaluating the survival was same as for calcium cross linked matrices. However the plates were stored in various RH conditions to test the potential application of synbiotic matrices in wide range of products. Hence, jars of varying RH values (16%, 33%, 72%, 98%) were created using saturated solutions of lithium chloride, magnesium chloride, lithium acetate and potassium sulfate respectively [58] and the 96-well plates were stored within these jars (lids not tightly sealed) for 3 weeks at 4ºC.

2.2.5. Modeling Bacterial Growth

The plate counts obtained from the each of the specific time intervals were used to evaluate growth characteristics (lag time, growth rate and maximum population density). The Gompertz function [52] described below was fitted to the growth data sets using non-linear regression (SAS version 9.2, TS Level 2M3). This mathematical model is a function of time and is given by the equation:

$$L(t) = \log N = A + C \exp\{-\exp[-B(t-M)]\}$$

Where, A = asymptotic log-count as time, 't' decreases indefinitely, C = asymptotic amount of growth that occurs as t increases indefinitely, B = relative growth rate at M, and M is the time at which the absolute growth rate is a maximum.

Table 7: Growth Characteristics determined from Gompertz Model

Lag Time (h)	Growth Rate (μ) (log(CFU/ml)/h)	Max Population Density (CFU/ml)	Generation Time (h)
M- 1/B	BC/e, where e=2.7182	B	log (2) e /(B*C)

The control used in this study was the growth characteristics of bacteria (not stored in any matrix and t = 0) grown in BHI broth for 48 h. The growth characteristics were separated using the pair-wise least square deviation technique ($P<0.05$). A score card was developed to evaluate the net difference between the benefits of POS, FOS, inulin and alginate fibers on the storage of bacteria. A point system (Table 2) was used to evaluate the change in the growth characteristics of bacteria between the control and the end of storage period (4th week). Reduction in lag time, increase in growth rate and maximum

population density is considered a positive shift (assigned a score of +1) in growth characteristics and vice versa for a negative shift (score of -1).

Table 8: Criteria for scoring

Growth Character	Shift in letters (Direction: Control to 4th week)	Points Assigned
Growth Rate & MPD	C to A, C to BA, C to B, B to A	1
	A to C, A to B, A to BC, B to C	-1
Lag Time	C to A, C to BA, C to B, B to A	-1
	A to C, A to B, A to BC, B to C	1
For all characteristics	C to CB, B to BA, A to AB, B to BC	0

2.2.6. Short Chain Fatty Acid Analysis

The 1 milliliter samples were centrifuged at 13,000 x g for 10 min [53, 54] and filtered (0.22 µm) prior to high performance liquid chromatography (HPLC) analysis. An autosampler was programmed to inject 20 µl of the sample onto a HPLC system that included a refractive index detector (Shimadzu RID-10A), an Aminex HPX-87H column (300 x 7.8mm, Bio-Rad, Hercules, CA) maintained at 40ºC, and a Cation H micro-guard column (30 x 4.6 mm, Bio-Rad, Hercules, CA). The eluent was 5 mM H_2SO_4 at a flow rate of 0.6 ml/min. The data was collected and analyzed using a Chromeleon (ver. 6.8) workstation. Quantification of samples utilized calibration curves of lactic, acetic, propionic and butyric acids (0.05-0.1M) [55]. The experiments were repeated in replicates. Differences in the SCFA data was tested for significance ($P<0.05$) using ANOVA (SigmaPlot 11.0, Systat Software Inc) using the Holm-Sidak method of pairwise multiple comparison.

2.2.7. Scanning Electron Microscopy

The matrix were frozen in liquid nitrogen and fractured using a cold scalpel blade followed by sputter coating with gold. Some samples were analyzed directly without freeze fracture. The samples were examined with a Quanta 200 FEG environmental scanning microscope (FEI Co., Inc., Hillsboro, OR) operated in the high vacuum, secondary electron imaging mode.

3. Results and Discussions

3.1. Synbiotic Matrices

We developed two variants of the matrices, with and without calcium fixation. Due to some inconclusive mechanism dry pellets were formed after about 2-3 days of storage when probiotic bacteria was inoculated in calcium fixed matrices and soft gel like matrices were formed in absence of calcium fixation as shown in the Figure 3. Based on existing literature, this observation can be attributed to a combination of lowered absorption of water, lower bound water retention and evaporation of free water. Higher the concentration of calcium chloride (0.01M – 0.1M) stronger will be the cross linking in the matrix and lower will be the water absorption capacity of alginate matrices [56]. The pH of the fermented media added to the matrices is acidic which also might have led to proton-calcium ion exchange forming insoluble acid gels with low water retention capabilities [57].
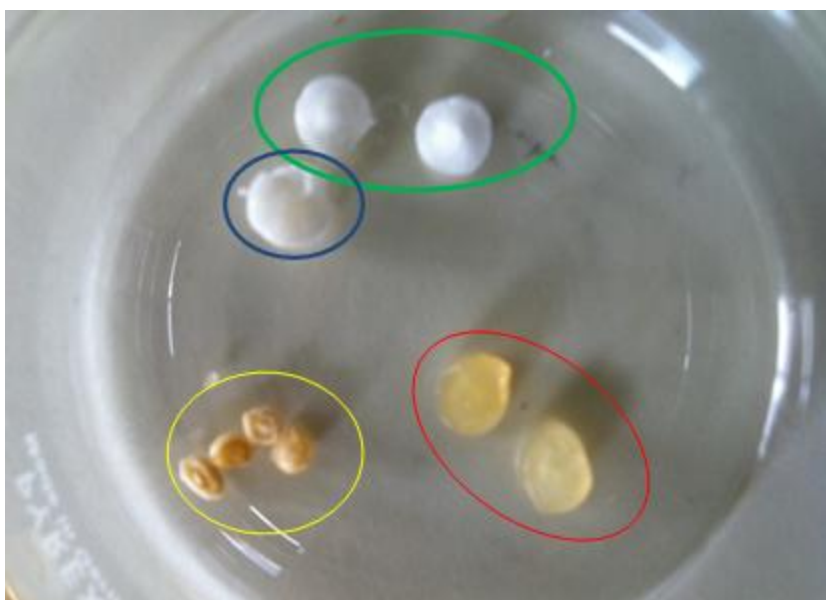
Figure 3: Matrices (Uninoculated / inoculated matrices with (Blue, Yellow) / without (Green, Red) calcium fixation respectively)

In our study, the 0.045 M calcium chloride fixed matrix showed very low water retention. In soft gels, absence of calcium made them relatively fragile and especially the matrix with POS broke apart during extraction from the wells of 96-well plate. Both matrices dissolved immediately in growth media (pH: 5.5-7) suggesting further optimization for an industrial application. Hence an optimal concentration of calcium chloride should be determined for achieving both structural integrity and hydration over storage conditions.

3.2. Scanning Electron Microscopy

The qualitative analysis of survival after storage of synbiotics in refrigeration conditions for 30 days was covered in the previous study by Hotchkiss et.al [36]. In this study, the long term effects of storage (4 months and refrigeration conditions) on the survival of bacteria were determined. The following strains of probiotics survived when stored in various prebiotic containing matrices.

Table 9: Dimensions of bacteria

Bacteria	Length (µm)
Lactobacillus acidophilus	1.2 to 3
Bifidobacterium longum	0.3 to 0.8
Bifidobacterium reuteri	6 to 6.8

Table 10: Synbiotic Matrices Analysis

Fiber	Pore Size		Appearance			Presence of Bacteria	
	IS(µm)	OS (µm)	Magnification	Inner Surface	Outer surface	Inner Surface	Outer surface
Alginate	95.8-98.3	0.2-0.5	5000X	Irregular honeycomb like cavities	Rough, Irregular	None	Scattered
POS	65.4-78.5	Nil	5000X	Smaller honeycomb like cavities	smooth	Few patches	Scattered
FOS	86.4-92.3	0.46-0.52	5000X	Irregular compartments	Smooth and layered	Few scattered	Well distributed patches
Inulin	90.31-108.38	0.58-0.72	5000X	Regular honey comb like cavities	Patches of tightly bound worm like structures	Thick outgrowth, lots of patches	Few patches

Although the amount of bacteria that survived within these matrices is very low (2-4 logs) they were metabolically active and reached 8 logs in 24 hours of growth. These matrices are not suitable for any commercial application that require 4 months of shelf life, as previously discussed 7-8 logs of bacteria is required for a health benefit [10, 28]. This analysis shows the importance of using prebiotic fibers for prolonging the survival as not much bacteria survived in alginate matrices. Most of the bacteria were found in the internal cavities of the matrices, the reasons for which can be further investigated.

SEM images of calcium fixed matrices are as follows:

Figure 4: Calcium Alginate Matrix without any prebiotic
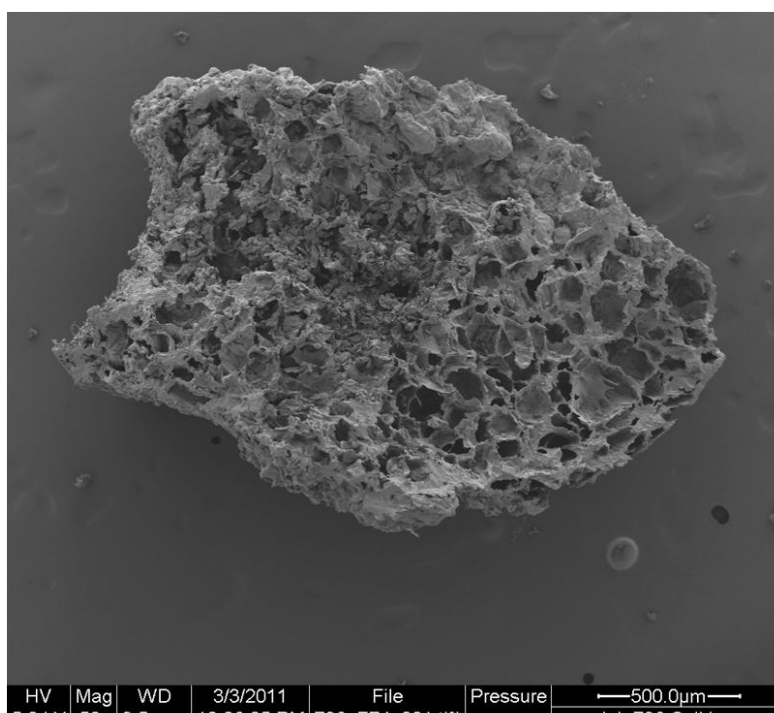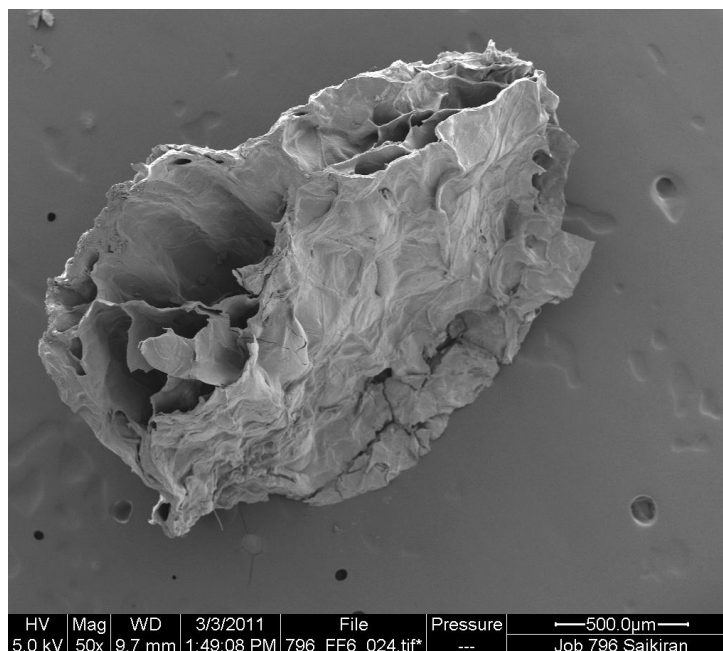
Figure 5: Internal cavities of FOS-Ca Alginate matrix

Figures 4, 5 show the matrices with internal cavities. Alginate matrix without prebiotic fiber did not support survival of bacteria during 4 months of storage. This was confirmed with no growth in MRS broth even after 24 hours. The cavities in Figure 5 have shown to support the survival of colonies that were also metabolically active.
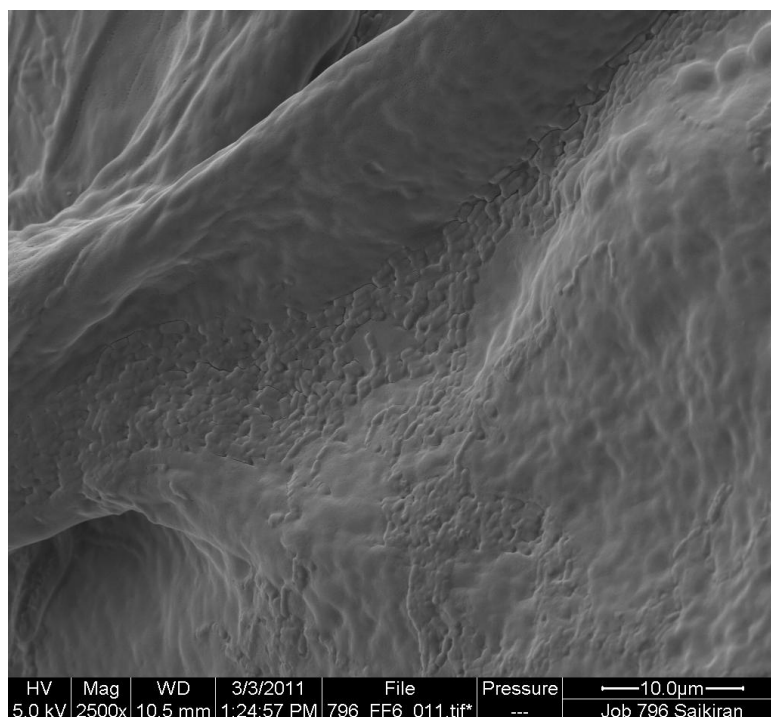
Figure 6: Strain 3300 on the outer surface of POS-Ca-Alg matrix
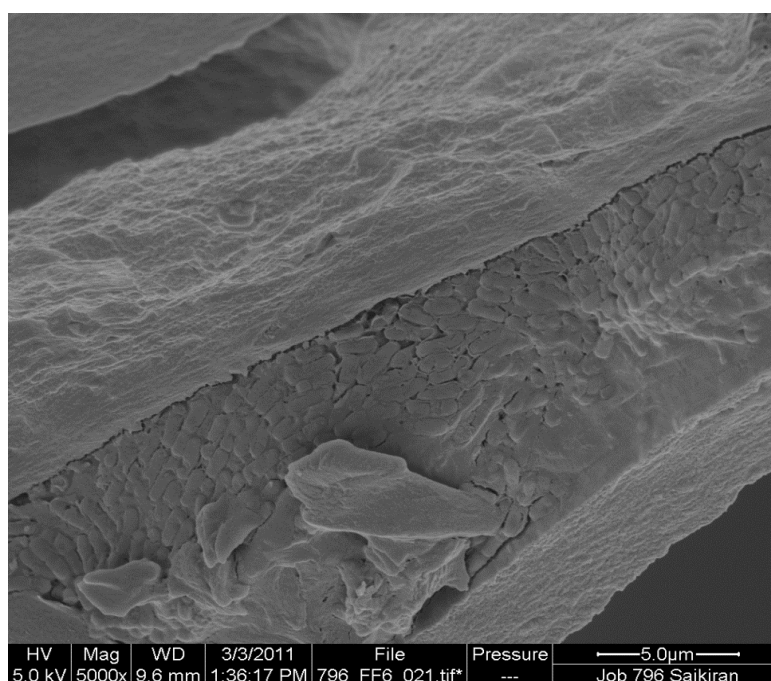
Figure 7: Strain 2141 in a section of FOS-Ca-Alg

Bifidobacterium species were found to survive in POS and FOS matrices even after 4 months of storage (Figures 6, 7). They were found on both external and internal layers of the matrix.
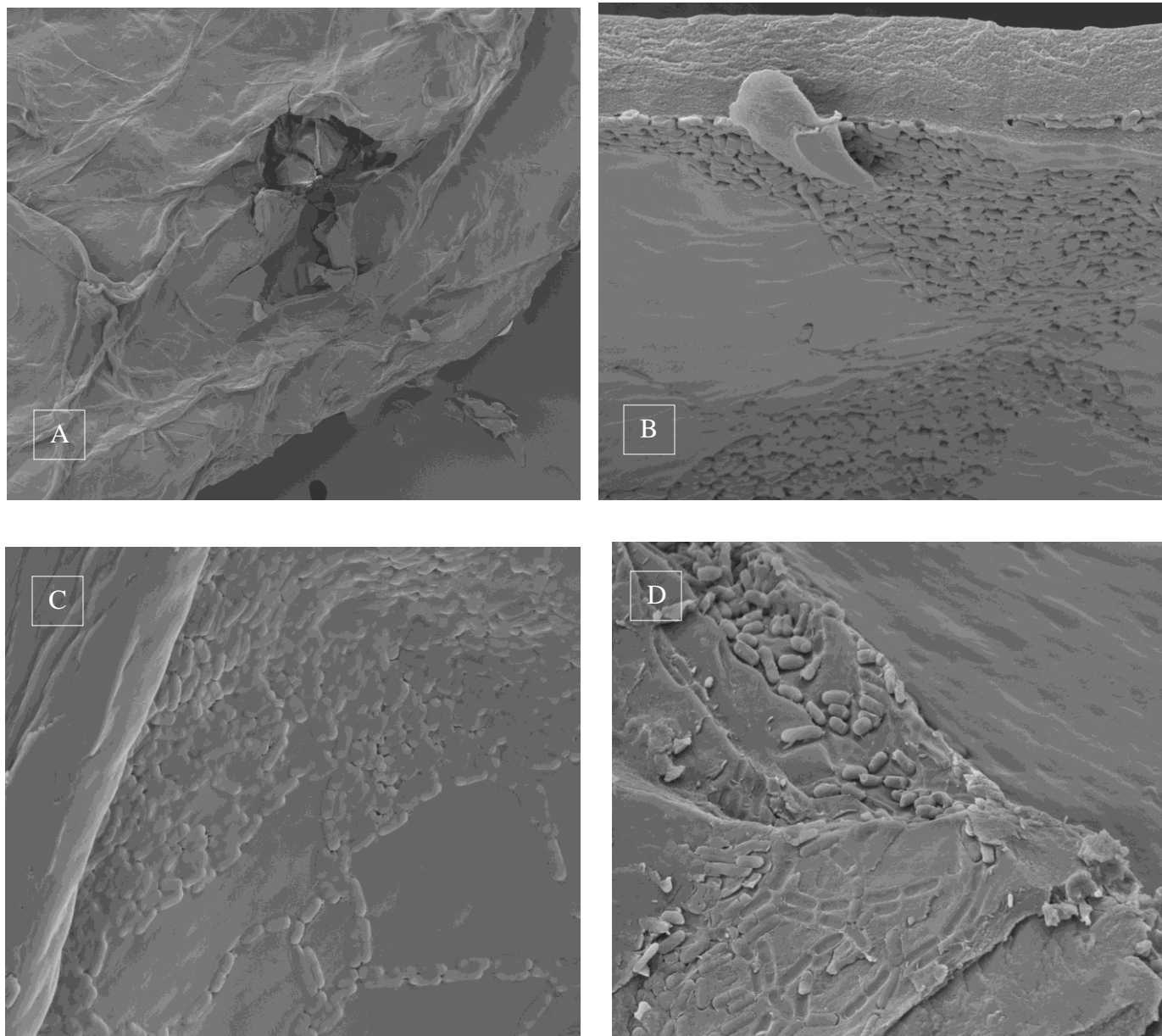
Figure 8: Images A-D: SEM images of Inulin-Alginic Acid-Calcium Matrix filled with Strain 1426 after 4 months of storage at 4ºC

Lactobacillus acidophilus was found in all the prebiotic matrices with abundance in inulin matrix (Figure 8). Lawns of bacteria were present inside a pore located on the surface of the matrix.

SEM images of non-calcium fixed matrices are as follows:
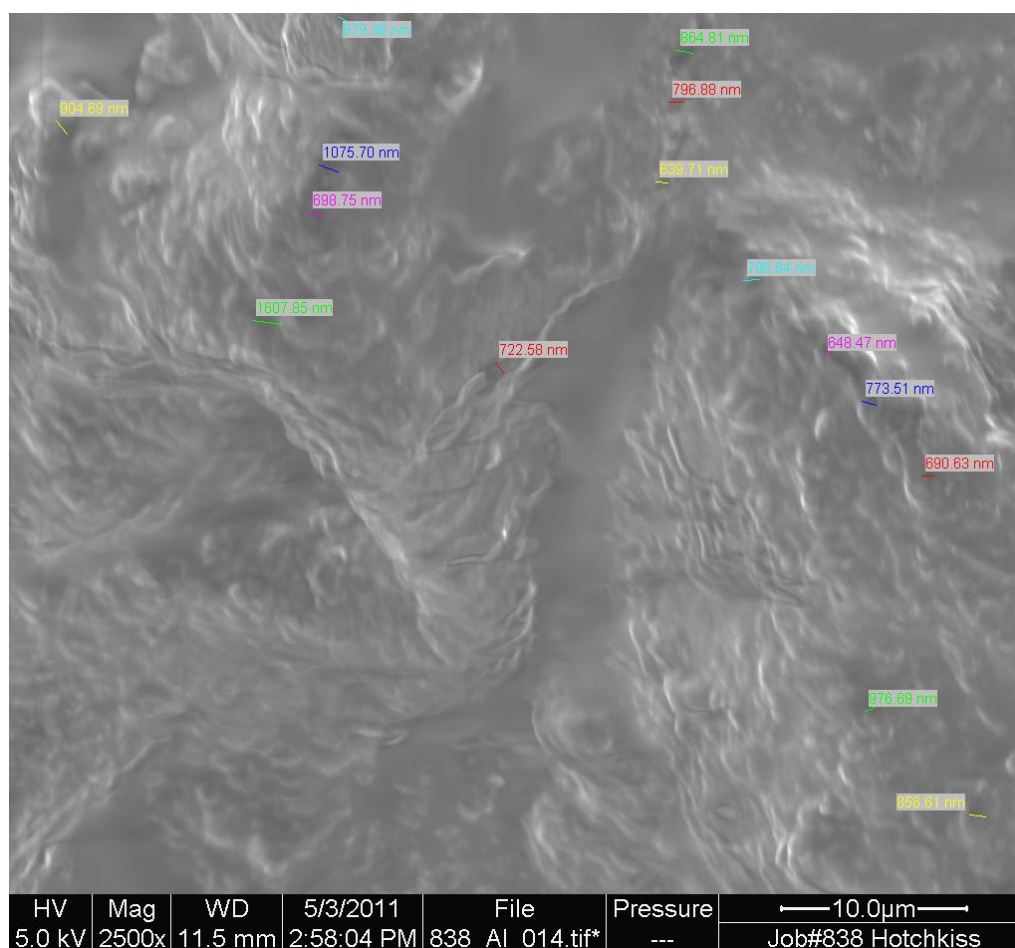
Figure 9: Bacteria on surface of inulin-alginate matrix
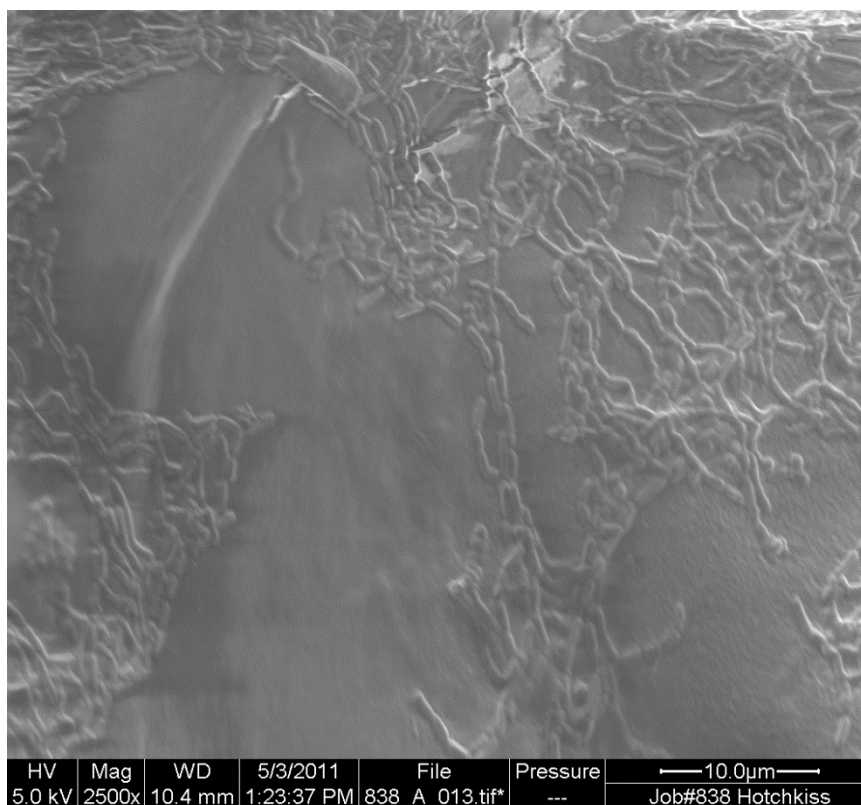
Figure 10: Bacteria on surface of Alginate Matrix
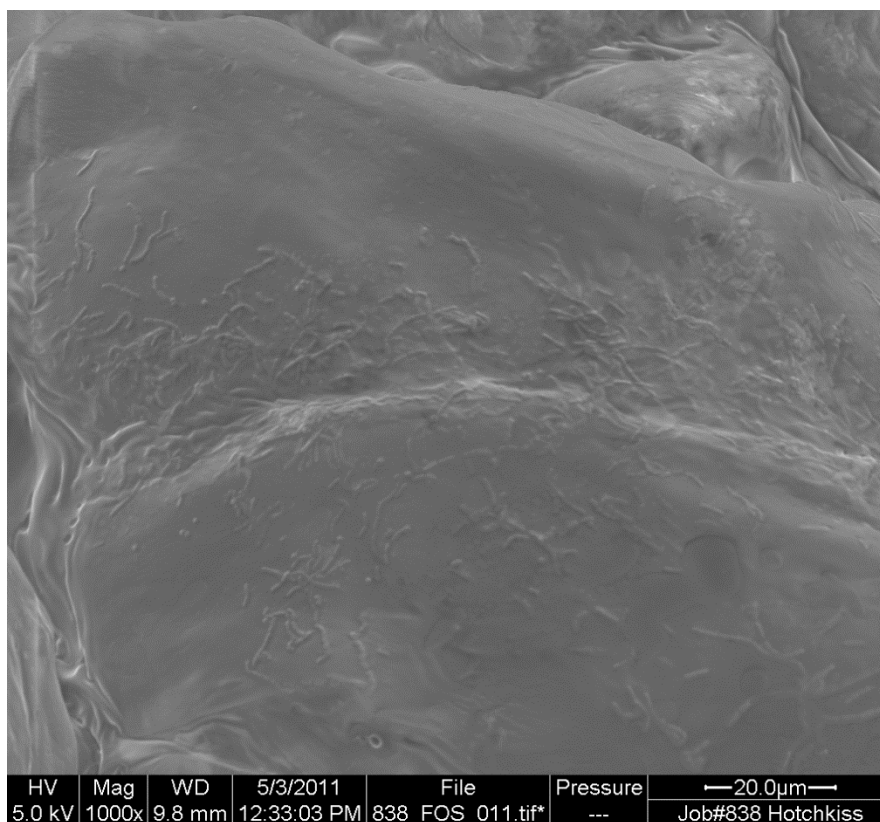
Figure 11: Bacteria on surface of FOS matrix
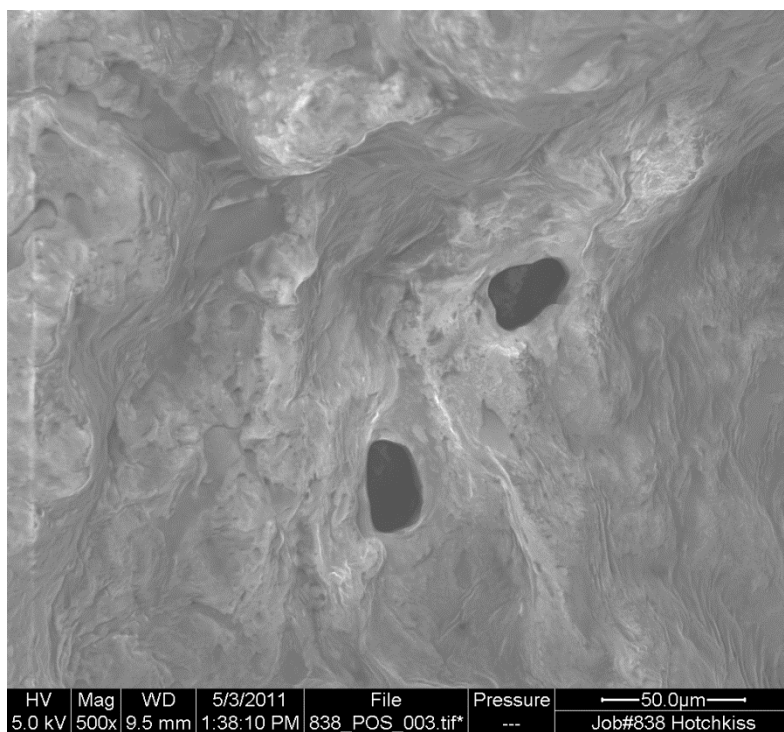
Figure 10: Bacteria present (figure below) inside the pores of POS matrix (figure above)
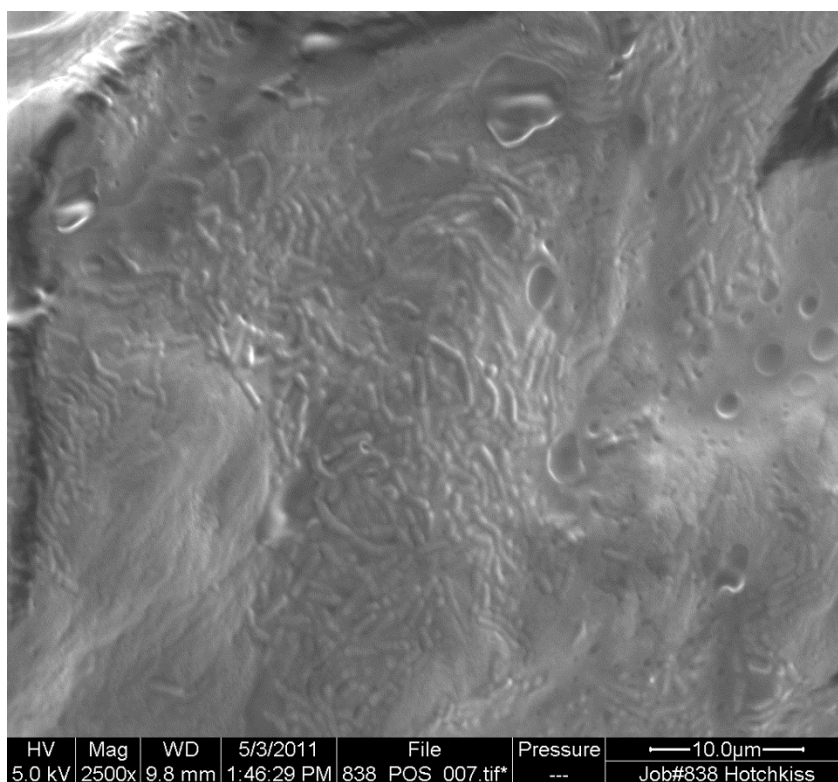
Non-calcium matrices also showed survival of different strains of probiotic organisms inside them after 2 months of refrigerated storage. However bacteria were also found in alginate matrices.

3.3. Survival studies of probiotic bacteria

3.3.1. Calcium cross linked matrices

The protective effect of POSA, FOSA and IA relative to alginate fibers on the survival of probiotic bacteria at 4°C under aerobic conditions was evaluated. Significant numbers of bacteria (0 hour anaerobic plate counts) survived after 7, 14, 21, 28 days of storage (Table 11). In general, Lactobacillus species had better survival in the prebiotic fibers under refrigerated aerobic conditions compared to alginate, a non-prebiotic control. Significantly higher survival of *Lactobacillus acidophilus* was observed in POSA after 7 and 28 days, FOSA after 7 days and IA after 28 days of storage compared to a solely calcium alginate matrix (Table 11). *Lactobacillus reuteri* responded well in POSA (significantly higher survival 7-21 days), FOSA (14-21 days) and IA after 28 days of storage compared to calcium alginate. Bifidobacteria did not survive as well in the prebiotic matrices compared to growth in calcium alginate (Table 11). Except for *Bifidobacterium breve* after 21 days of storage in FOSA and IA, if a significant difference in survival was observed, then it was lower in the prebiotic matrix compared to calcium alginate alone. Bifidobacteria are obligate anaerobes, which may have contributed to their lower survival under the refrigerated aerobic storage conditions in the presence of prebiotics compared to Lactobacilli, which are facultative anaerobes.

Table 11: Survival of probiotic bacteria (log10 (CFU/100ml)) in calcium fixed prebiotic matrices during 28 days of storage at 4°C under aerobic conditions

Bacteria Strain	Storage	POSA	FOSA	IA	Alginate
Lactobacillus	7	5.29 ± 0.02^a	5.39 ± 0.03^a	4.09 ± 0.12	4.52 ± 0.07
acidophilus	14	5.01 ± 0.36	4.99 ± 0.48	4.49 ± 0.31	3.71 ± 0.29
	21	4.27 ± 0.06	4.93 ± 0.16	4.34 ± 0.06	3.54 ± 0.65
	28	4.69 ± 0.13^d	3.95 ± 0.80	4.67 ± 0.09^d	3.36 ± 0.01
Lactobacillus	7	5.76 ± 0.04^a	4.09 ± 0.12	3.85 ± 0.21	4.60 ± 0.14
reuteri	14	5.02 ± 0.14^b	5.86 ± 0.05^b	4.38 ± 0.60	4.29 ± 0.07
	21	4.38 ± 0.01^c	4.64 ± 0.08^c	4.35 ± 0.60	4.08 ± 0.04
	28	4.45 ± 0.40	4.67 ± 0.31	4.87 ± 0.07^d	4.71 ± 0.09
Bifidobacterium	7	2.00 ± 0.00	2.28 ± 0.04	2.00 ± 0.00	2.00 ± 0.00
breve	14	2.57 ± 0.81	2.00 ± 0.00^b	2.60 ± 0.43	2.97 ± 0.10
	21	2.64 ± 0.90	3.60 ± 0.06^c	3.57 ± 0.11^c	2.85 ± 0.00
	28	2.00 ± 0.00	2.92 ± 0.63	2.00 ± 0.00	3.07 ± 0.16
Bifidobacterium	7	4.73 ± 0.03^a	5.75 ± 0.20	5.70 ± 0.20	5.38 ± 0.06
longum	14	3.10 ± 0.56	4.44 ± 0.03^b	4.55 ± 0.03^b	5.05 ± 0.04
	21	4.14 ± 0.03^c	4.35 ± 0.07^c	4.72 ± 0.02	5.11 ± 0.14
	28	4.07 ± 0.07^d	4.45 ± 0.10^d	4.91 ± 0.11	5.32 ± 0.03

[a]Significantly different (P<0.05) from Calcium Alginate after 7 days of storage

[b] Significantly different (P<0.05) from Calcium Alginate after 14 days of storage

[c] Significantly different (P<0.05) from Calcium Alginate after 21 days of storage

[d] Significantly different (P<0.05) from Calcium Alginate after 28 days of storage

The overall amounts of bacteria that survived in these matrices range from 3.5 to 5.5 logs for *Lactobacillus* species and 2-5 logs for *Bifidobacterium* species. The survival levels are not sufficient for a commercial application. The physical state and water activity of the matrix are the major factors that affect the survival of bacteria. A definitive relationship between the physical characteristics of matrix and probiotic bacteria is not well understood. Sorption isotherms for probiotic bacteria, if established, can throw more light on the observed phenomenon [59].

3.3.2. Non-calcium cross linked gel like matrices

3.3.2.1. Survival Study

All the non-calcium cross-linked matrices were inoculated with 4 species of bacteria and stored at 4ºC for 3 weeks. After each week of storage, the matrices were evaluated for bacterial numbers (plate counts) and the survivability was compared. There was no significant difference in the survival of bacteria in any of the prebiotic fibers over 3 weeks of storage (Table 12).

Table 12: Survival of probiotic bacteria (log10 (CFU/ml)) in synbiotic matrices (without calcium chloride) during 21 days of storage at 4ºC under aerobic conditions

Bacteria Strain	Storage Time (days)	POSA	FOSA	IA	Alginate
L. acidophilus	21	7.45 ± 0.07^c	7.54 ± 0.18^c	7.65 ± 0.1^c	4.89 ± 0.09
L. reuteri	21	7.51 ± 0.15^c	7.72 ± 0.13^c	7.65 ± 0.19^c	5 ± 0.07
B. breve	21	7.42 ± 0.18^c	7.76 ± 0.15^c	7.65 ± 0.18^c	4.75 ± 0.11
B. longum	21	7.29 ± 0.17	7.51 ± 0.11	7.48 ± 0.15	7.19 ± 0.1

^{c} Significantly different (P<0.05) from Alginate after 21 days of storage

However a steady decline in the bacteria counts was observed in alginate matrices except for *Bifidobacterium longum*. These results also demonstrate a much higher survival of bacteria than the ones stored in calcium cross linked matrices (Table 11). The possible reason for this phenomenon can be attributed to the physical state of both matrices. Studies have shown a positive effect of glassy state of a polymer on the survival of bacteria. In glassy state, diffusion related deterioration reactions are reduced. Moisture transactions during storage fluctuate due to desorption, adsorption, and glass transition phenomena [59]. The matrix wouldn't have too many transactions if a glassy state is created and maintained by storing at temperatures below glass transition temperature, Tg. Studies have shown maximum survival of bacteria if the storage temperature, T was at

least 30-50 °C lesser than Tg [59]. Calcium cross linking rendered the fibers a dry porous physical form and hence very negligible moisture for the survival of bacteria. However, without calcium cross linking, the fibers had a gel like physical form with more moisture for the survival of bacteria. The focus of this study demonstrated that water activity was important for survival of probiotic bacteria under refrigerated aerobic storage conditions. However, after the ingestion, the synbiotic should also possess structural integrity to survive the acidic gastric conditions. Therefore, a balance must be found between a hydrated synbiotic matrix that will improve shelf life and a protective barrier that will deliver at least 10^7 CFU/ml of probiotic bacteria to the colon.

3.3.2.2. Effect of relative humidity on survival of bacteria

In order to use these matrices in various products, the effect of relative humidity on the survival of bacteria within these matrices should be evaluated. Hence various RH levels (16%, 33%, 72%, 98%) were investigated. The results from this study indicated that RH did not have any significant effect on the reduction of bacteria over time.

Figure 11: Reduction of bacteria v/s RH levels
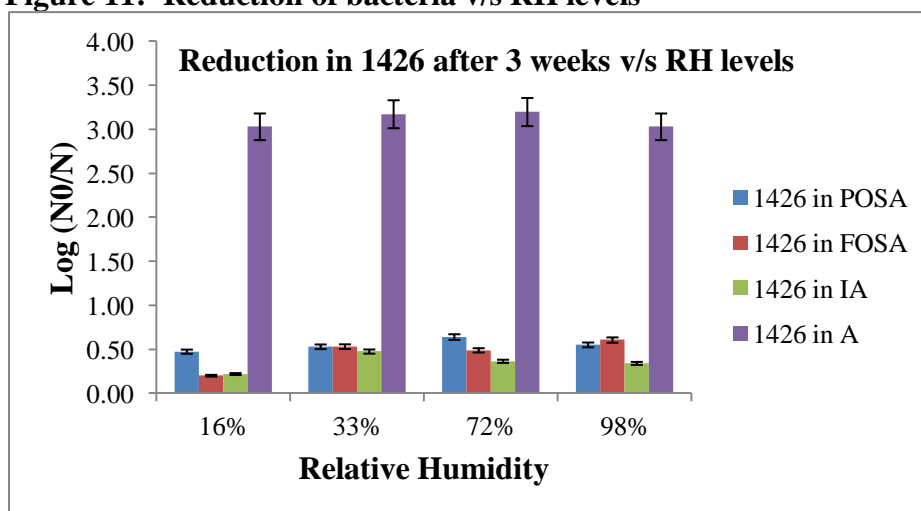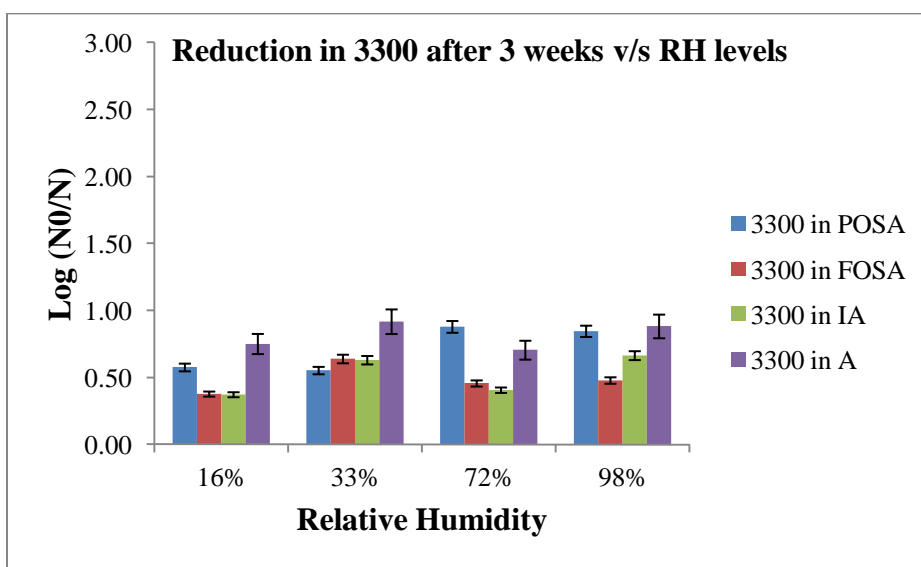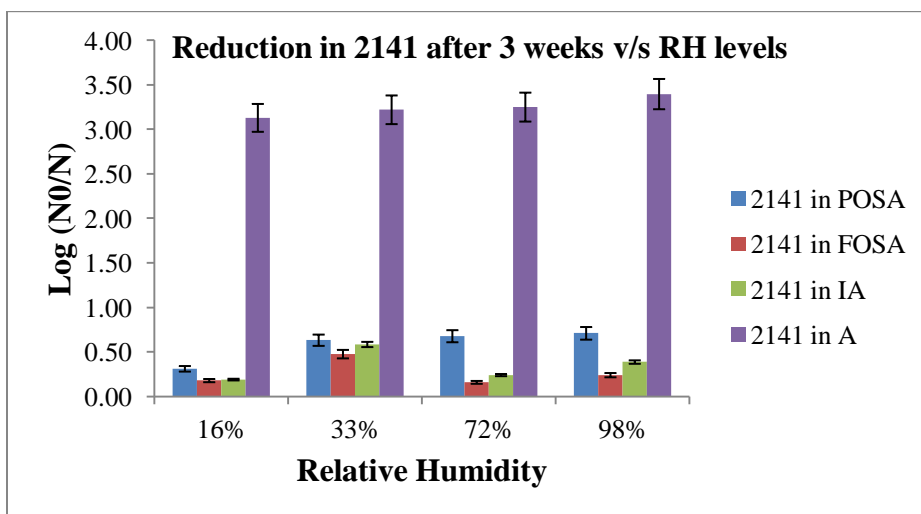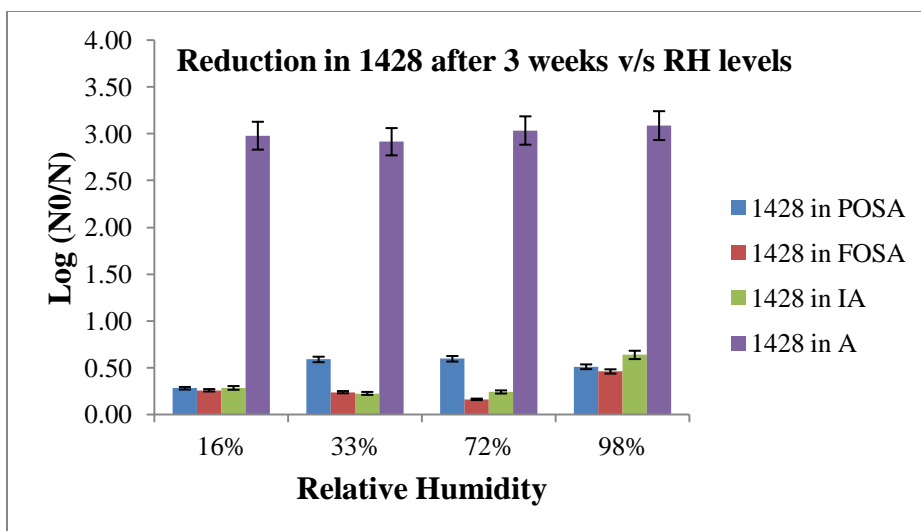
3.4. Growth characteristics of bacteria stored in calcium fixed synbiotic matrices

The Gompertz model growth characteristics of probiotic bacteria after 4 weeks of storage in different calcium fixed synbiotic matrices were evaluated (Table 13). Analysis of variance was performed between 1426, 1428 Lactobacillus strains and 2141, 3300 Bifidobacterium strains for each individual growth character. The growth characters of bacteria stored in fibers were compared immediately with the control and assigned a letter of the alphabet based on the differences. Growth characteristics were influenced by the presence of prebiotics for both Bifidobacteria and Lactobacilli (Table 13). The growth rate of bacteria stored in different fibers remained the same throughout. There was also an increase in maximum population density observed for *L. reuteri* in POSA, FOSA, IA and A. There were no significant changes in generation time for any probiotic stored in any of the fibers (Table 13).

Table 13: Growth Characteristics of probiotic bacteria after 28 days of 4ºC aerobic storage in different fibers (mean ± standard error)

Treatment	GR	GR_S		Lag	Lag_S		GT	GT_Se		MP	MPD_S	
26POSA	1.0	4.46	A	8.5	10.12	A	0.2	1.28	A	8.9	0.42	A
1426*	0.4	0.04	A	9.6	0.90	A	0.7	0.07	A	9.2	0.25	A
Score			0			0			0			0
26FOSA	0.4	0.06	A	2.7	1.06	B	0.7	0.10	A	9.4	0.43	A
1426*	0.4	0.04	A	9.6	0.90	A	0.7	0.07	A	9.2	0.25	A
Score			0			1			0			0
26IA	0.5	0.16	A	7.4	1.36	A	0.5	0.16	A	9.0	0.31	A
1426*	0.4	0.04	A	9.6	0.90	A	0.7	0.07	A	9.2	0.25	AB
Score			0			0			0			0
26A	0.4	0.12	A	7.3	1.46	A	0.6	0.15	A	9.0	0.39	A
1426*	0.4	0.04	A	9.6	0.90	A	0.7	0.07	A	9.2	0.25	A
Score			0			0			0			0
28POSA	0.5	0.10	A	6.5	0.93	A	0.5	0.10	A	9.1	0.26	A
1428*	0.2	0.02	A	9.3	0.65	A	1.1	0.07	A	7.9	0.14	B
Score			0			0			0			1

Sample	GR	S		GT	S		Lag	S		MPD	S	
28FOSA	0.4	0.08	A	5.6	1.17	A	0.7	0.13	A	9.3	0.34	A
1428*	0.2	0.02	A	9.3	0.65	A	1.1	0.07	A	7.9	0.14	B
Score			0			0			0			1
28IA	0.3	0.06	A	2.7	1.28	B	0.8	0.13	A	8.9	0.43	A
1428*	0.2	0.02	A	9.3	0.65	A	1.1	0.07	A	7.9	0.14	B
Score			0			1			0			1
28A	0.4	0.17	A	5.5	2.18	A	0.6	0.25	A	8.8	0.63	A
1428*	0.2	0.02	A	9.3	0.65	A	1.1	0.07	A	7.9	0.14	B
Score			0			0			0			1
21POSA	0.5	0.16	A	-	1.68	A	0.5	0.14	A	6.8	1.33	A
2141*	0.3	0.18	A	3.4	3.30	A	0.8	0.48	A	7.7	0.94	A
Score			0			0			0			0
21FOSA	0.3	0.11	A	1.4	2.70	A	0.8	0.27	A	7.7	1.02	A
2141*	0.3	0.18	A	3.4	3.30	A	0.8	0.48	A	7.7	0.94	A
Score			0			0			0			0
21IA	0.4	0.10	A	1.0	1.35	A	0.6	0.12	A	8.0	0.67	A
2141*	0.3	0.18	A	3.4	3.30	A	0.8	0.48	A	7.7	0.94	A
Score			0			0			0			0
21A	0.4	0.09	A	3.0	1.22	A	0.6	0.11	A	8.4	0.50	A
2141*	0.3	0.18	A	3.4	3.30	A	0.8	0.48	A	7.7	0.94	A
Score			0			0			0			0
33POSA	0.5	0.25	A	2.1	2.47	A	0.6	0.30	A	7.9	1.08	A
3300*	0.4	1.11	A	7.9	7.08	A	0.6	1.48	A	7.3	1.34	A
Score			0			0			0			0
33FOSA	0.4	0.17	A	-	2.95	B	0.6	0.28	A	8.1	2.02	A
3300*	0.4	1.11	A	7.9	7.08	A	0.6	1.48	A	7.3	1.34	A
Score			0			1			0			0
33IA	0.3	0.10	A	-	1.90	B	0.8	0.22	A	8.4	0.89	A
3300*	0.4	1.11	A	7.9	7.08	A	0.6	1.48	A	7.3	1.34	A
Score			0			1			0			0
33A	0.5	0.08	A	2.2	0.70	A	0.5	0.09	A	8.4	0.32	A
3300*	0.4	1.11	A	7.9	7.08	A	0.6	1.48	A	7.3	1.34	A
Score			0			0			0			0

*Control: Bacteria before inoculation into matrices, A = Alginate, POSA = Pectic Oligosaccharide + Calcium Alginate, FOSA = Fructo Oligosaccharide + Calcium Alginate, IA = Inulin + Calcium Alginate, 26 = *Lactobacillus acidophilus*, 28 = *Lactobacillus reuteri*, 21 = *Bifidobacterium breve*, 33 = *Bifidobacterium longum*; GR - growth rate, Lag - lag time, GT - growth time, MPD - maximum population density, S - standard error. Common letters are not statistically different (P <0.05) using ANOVA.

Table 14: Scores for fiber performance

Strain	POSA	FOSA	IA	Alginate
1426	0	1	0	0
1428	1	1	2	1
2141	0	0	0	0
3300	0	1	1	1
Total	1	3	3	2

The positive effects of prebiotic fiber were found to be strain specific and statistically FOSA, IA had a better overall effect than POSA. Since the required cutoff values for growth characters for an intended positive effect is not known, the magnitudes of growth characteristics (lag time, growth rate and maximum population density) were not taken into account while evaluating the performance of the fibers. Irrespective of the treatment there is no depreciation in the growth characteristics of these organisms.

3.5. Short chain fatty acid analysis

The difference in bacterial strains ability to produce short chain fatty acid during their 48 hour growth period was analyzed (Table 15). These bacteria were extracted from the calcium fixed synbiotic matrices. All strains that were stored in each of the fibers produced increasing amounts of lactic acid during anaerobic growth (Table 15). Lactobacillus species produced higher amounts of acetic, propionic and butyric acids than Bifidobacterium for most of the time intervals. *Bifidobacterium longum* stored in alginate produced higher amounts of lactic acid than the lactobacillus species after 24, 48 hours of incubation. In all cases, the levels of butyric, acetic acids produced were very low and the lactic acid levels produced in this study were very high when compared to previous studies [55]. The system used in previous study was mixed fecal culture slurry which contributes to higher SCFA values as compared to pure probiotic cultures. The SCFA

production by probiotic organisms is very important; however the effect of individual organic acid levels on a specific health benefit is not well established. A large standard deviation was observed in the organic acid levels (24, 48 hours) which indicate that there was an effect of storage on acid production ability of organisms and also some of the volatiles might be lost during sample processing. However, a definitive relationship cannot be established until each strain's fermentation capabilities are well determined.

Mean concentration (mM) of organic acids produced after storage in fibers over 4 weeks						
Fiber	Time	Strain	Lactic Acid	Acetic Acid	Propionic Acid	Butyric Acid
	24	1426	41.65±6.47	7.83±1.58	13.51±1.24[a]	0.86±0.29
POSA		1428	39.35±9.30	7.93±0.84	13.89±1.61[a]	0.83±0.61
		2141	37.47±12.24	6.44±2.38	8.45±3.93[b]	0.00
		3300	40.84±12.88	8.02±6.97	8.30±3.91[b]	0.00
	48	1426	46.92±3.99	8.61±1.92[a]	11.43±6.66	1.37±0.68
		1428	44.76±5.92	8.63±0.81[a]	15.23±1.24[a]	0.96±0.37
		2141	39.49±12.84	5.22±2.74[b]	8.11±2.06[b]	0.00
		3300	47.44±9.16	5.01±1.45[b]	7.35±4.12[b]	0.00
	24	1426	39.81±7.28	7.90±0.65	13.62±1.38[a]	0.87±0.69
FOSA		1428	34.42±10.99	7.38±0.62	13.09±1.32[a]	1.52±0.94
		2141	35.92±13.08	6.24±3.39	8.34±4.11[b]	0.00
		3300	43.97±15.50	7.80±6.17	8.30±4.07[b]	0.00
	48	1426	46.05±3.90[a]	8.45±0.83[a]	15.34±1.36[a]	1.20±0.74
		1428	48.11±1.58[a]	8.41±0.77[a]	15.63±0.55[a]	1.08±0.47
		2141	34.84±9.05[b]	4.90±2.85[b]	5.49±3.43[b]	0.00
		3300	46.91±10.29[a]	4.08±1.76[b]	7.36±2.53[b]	0.00
	24	1426	25.96±10.71[a]	7.45±0.22[a]	12.54±0.58[a]	0.88±0.49
IA		1428	28.68±8.45[a]	7.53±0.37[a]	12.53±0.84[a]	1.20±0.74
		2141	37.59±16.22[a]	6.52±2.19[b]	7.89±3.39[b]	0.00
		3300	44.53±13.84[b]	4.17±2.00[b]	7.49±2.70[b]	0.00
	48	1426	47.48±2.00[a]	8.51±0.84	15.38±0.38[a]	1.12±0.66
		1428	48.05±0.60[a]	8.55±0.87	15.09±0.66[a]	1.02±0.43
		2141	30.08±10.26[b]	5.95±2.70	7.42±2.68[b]	0.00
		3300	47.10±13.44[a]	5.89±3.05	7.88±3.39[b]	0.00
	24	1426	20.92±14.73[a]	7.23±0.68[a]	12.32±0.58[a]	1.37±1.13
Alginate		1428	23.28±10.24[a]	8.28±1.07[a]	12.42±0.48[a]	0.99±0.40
		2141	33.11±13.68	6.15±3.26[b]	8.33±3.88	0.00
		3300	43.08±15.44[b]	5.27±2.15[b]	7.52±2.83[b]	0.00
	48	1426	23.28±10.24[a]	8.44±1.32[a]	14.70±1.33[a]	1.15±0.67
		1428	47.45±2.01[b]	9.30±0.86[a]	15.07±0.72[a]	1.08±0.68
		2141	33.98±12.32	4.85±2.75[b]	7.52±2.68[b]	0.00
		3300	44.65±14.43[b]	4.03±2.00[b]	7.33±2.67[b]	0.00

Table 15: Short Chain Fatty Acid Analysis

a,b,c,d Significantly different acid concentration from other strain with different alphabet within a column. All values mentioned are mean ± standard deviation of 4 weeks of samples with replicates.

a, b, c, d are results of ANOVA with n=8 and they are separated using Holm-Sidak method of pairwise multiple comparison with P<0.05.

4. Conclusions

There are various factors that affect the commercialization of synbiotic matrices. In this study the primary focus was on bacteria related factors such as their survivability, growth characteristics and short chain fatty acid production abilities after storage. The objectives of this research were to produce various synbiotic systems and perform a technical feasibility analysis for commercializing them.

In this regard, we developed two kinds of synbiotic matrices and calcium cross linking rendered the first system drier than the other. The overall amounts of bacteria that survived in calcium fixed matrices varied from 3.5 to 5.5 logs for *Lactobacillus* species and 2-5 logs for *Bifidobacterium* species, which is insufficient for a commercial application. The growth characteristics of bacteria after revival from storage were same or better than the bacteria prior to storage. These bacteria were also capable of producing short chain fatty acids throughout the storage period.

A much higher level (7-7.5 logs) of bacteria survived in non-calcium cross linked synbiotic matrices, irrespective of the fiber and bacteria strain type, making this system ideal for a commercial application. We could also expect the growth characteristics and

short chain fatty acids levels of these bacteria to be higher or equal to the bacteria stored in calcium fixed matrices. Variation in relative humidity showed no negative effect on survival of bacteria when stored in these synbiotic matrices.

In conclusion, the bacteria stored in synbiotic matrices are still suitable for commercial applications but improvement in the structural stability of matrix and optimization of calcium cross linking is essential for furthering research in commercializing synbiotics.

5. Future Work

In this research we have dealt with testing the fitness of probiotic bacteria after storing in refrigerated aerobic conditions. It is a first successful step towards commercialization. Synbiotic matrix system can be part of multitude of potential food applications. The next steps towards commercialization can be divided into following categories:

1) **Structural stability to matrix**: Both calcium and non-calcium cross linked matrices are readily soluble in water and depending on the final application of this matrix there might be stability issues during the transit down the gastro-intestinal cavity. Previous studies related to encapsulation of probiotic strains of *Lactobacillus* and *Bifidobacterium* have shown high stability and improved survival in bile salts and acidic gut conditions [38-40, 43-45]. Developing a secondary layer of protection with other polysaccharides around the synbiotics can be a viable solution. This protection can also be provided by food matrices, as cheese and skim milk have shown at least 5 log better survival than without any form of encapsulation [46].

2) **Optimization of calcium cross linking**: Some of the gel like synbiotic matrices were very delicate and definitely need some kind of cross linking between the

polymers for structural integrity. However getting the right amounts of calcium is important as it affects the water absorption capacity of the polymers and hence low survival rates of bacteria. In order to establish the right amounts, a comprehensive sorption isotherm study should be performed for probiotic bacteria encapsulated in matrices.

3) **Further analysis:** Growth characteristics and SCFA production should be evaluated for bacteria stored in gel based synbiotic matrices. Even though the expected outcome is positive in these matrices it is good to perform a quick study and record the effects.

4) ***In Vivo* studies:** Animal studies can be performed on the developed synbiotic matrices to check the translation of all observed positive attributes of a synbiotic system into a health benefit.

6. References

1. Toni Steer, H. C., Kieran Tuohy and Glenn R. Gibson (2000). "Perspectives on the role of the human gut microbiota and its modulation by pro- and prebiotics." Nutrition Research Reviews (13): 229-254.
2. Taylor C Wallace, F. G., Karen Madsen, Michael D Cabana, Glenn Gibson, Eric Hentges, and Mary Ellen Sanders (2011). "Human gut microbiota and its relationship to health and disease." Nutrition Reviews **69**(7): 392-403.
3. Geoffrey A. Preidis, J. V. (2009). "Targeting the Human Microbiome with Antibiotics, Probiotics, and Prebiotics: Gastroenterology Enters the Metagenomics Era." GASTROENTEROLOGY(136): 2015-2031.
4. Francisco Guarner, A. G. K., James Garisch, Rami Eliakim, Alfred Gangl, Alan Thomson, Justus Krabshuis (2008). "Probiotics and prebiotics." World Gastroenterology Organisation.
5. C.N. Heenan, R.W. Hosken and G.H. Fleet (2004). "Survival and sensory acceptability of probiotic microorganisms in a non-fermented frozen vegetarian dessert." Lebensm.-Wiss. U.-Technol.(37): 461-466.
6. V.M. Sheehan et.al (2007). "Assessing the acid tolerance and the technological robustness of probiotic cultures for fortification in fruit juices." Innovative Food Sci. Emerg. Technol.(8): 279–284.
7. K.Y. Yoon, et.al (2006). "Production of probiotic cabbage juice by lactic acid bacteria." Bioresource Technology (97): 1427-1430.
8. K.Y. Yoon, et.al (2004). "Probiotication of tomato juice by lactic acid bacteria." J. Microbiology (42): 315-318.
9. J.K. Kadam (1989). "Critical reviews in food science and nutrition." Food Science(28): 348-400.
10. Shah, N. P. (2001). "Functional foods from probiotics and prebiotics." Food Technology(55): 46-53.
11. D. Charalapompoulos, C. Webb (2003). "Evaluation of the effect of malt, wheat and barley extracts on the viability of potential probiotic lactic acid bacteria under acidic conditions." International Journal of Food Microbiology (82): 133-141.
12. G. Kedia, R. W., H. Patel and S.S. Pandiella (2007). "Used of mixed cultures for the fermentation of cereal-based substrates with potential probiotic properties" Process. Biochem(42): 65-70.
13. M.H. Helland, T. W., J.A. Narvhus (2004). "Growth and metabolism of selected strains of probiotic bacteria, in maize porridge with added malted barley." International Journal of Food Microbiology (91): 305-313.
14. S. Ozdemir, A.Y. Kumral (2007). "A traditional Turkish fermented cereal food." Food Reviews International (23): 107-121.
15. S.D. Todorov, M. B., C. Guigas, U. Schillinger, I. Wiid, M.B. Wachsman, W.H. Holzapfel and L.M.T. Dicks (2008). "Boza, a natural source of probiotic lactic acid bacteria." J. Appl. Microbiol.(104): 465-477.
16. Y.C. Wang, R. C. Y., C.C. Chou (2002). "Growth and survival of bifidobacteria and lactic acid bacteria during the fermentation and storage of cultured soymilk drinks." Food Microbiology(19): 501-508.

17. T.D. Klingberg, L. A., K. Naterstad, D. Elsser, B.B. Bude (2005). "Identification of potential starter cultures for Scandinavian-type fermented sausages." <u>Int. J. Food Microbiol</u>(105): 415-431.

18. T. Luckow, C. D. (2004). "Which juice is healthier? A consumer study of probiotic non-dairy juice drinks." <u>Food Quality Preference</u>(15): 751-759.

19. Shah, N. P. (2000). "Probiotic Bacteria: Selective Enumeration and Survival in Dairy Foods." <u>Journal of Dairy Science</u> **83**: 894-907.

20. Yadira Rivera-Espinoza, Y. G.-N. (2010). "Non-dairy Food Products." <u>Food Microbiology</u> **27**(1): 1-11.

21. Gardiner, G. E., O'Sullivan, E., Kelly, J., Auty, M.A., Fitzgerald, G.F., Collins, J.K., Ross, R.P. and Stanton (2000). "Comparative survival rates of human-derived probiotic *Lactobacillus paracasei* and *L. salivarius* strains during heat treatment and spray drying." <u>Applied Environmental Microbiology</u> **66**: 2605-2612.

22. Selmer-Olsen, E., Sorhaug, T., Birkeland, S.E.,Pehrson (1999). "Survival of *Lactobacillus helveticus* entrapped in Ca-alginate in relation to water content, storage and rehydration." <u>Journal of Industrial Microbiology and Biotechnology</u> **23**(79-85).

23. Teixeira, P., Castro, H. and Kirby (1995). "Spray drying as a method for preparing concentrated cultures of *Lactobacillus bulgaricus*" <u>Journal of Applied Bacteriology</u> **78**: 456-460.

24. To, B. C. S., Etzel, M.R. (1997). "Spray drying, freeze drying, or freezing of three different lactic acid bacteria species." <u>Journal of Food Science</u> **62**: 576-587.

25. Sarkar, S. (2010). "Approaches for enhancing the viability of probiotics: A review." <u>Brit. Food J.</u> **112**: 329-349.

26. Zhao, Q., Lee, S.J., Mutukumira, A.N., Maddox, I., Shu, Q. (2011). "Viability and delivery of immobilized *Lactobacillus reuteri* DPC16 within calcium alginate gel systems during sequential passage through simulated gastrointestinal fluids." <u>Beneficial Microbes</u> **2**: 129-138.

27. Arnaud Picota, C. L. (2004). "Encapsulation of bifidobacteria in whey protein-based microcapsules and survival in simulated gastrointestinal conditions and in yoghurt." <u>International Dairy Journal</u> **14**(6): 505-515.

28. Ishibashi, N., Shimamura, S. (1993). "Bifidobacteria: research and development in Japan." <u>Food Technology</u> **47**: 126-135

29. Glenn R. Gibson, Karen P. Scott et.al, (2010). " Dietary prebiotics: current status and new definition" <u>Food Science and Technology Bulletin: Functional Foods</u> 7(1): 1-19

30. Yanbo Wang (2009). "Prebiotics: Present and future in food science and technology" <u>Food Research International</u> 42(1): 8-12

31. Luchansky, J.B. (2000). "Use of biotherapeutics to enhance animal well being and food safety" <u>Proceedings of the 6th International Feed Production Conference</u>

32. Ouwehand, A.C., Tiihonen, K., Saarinen, M., Putaala, H. and Rautonen, N. (2009). "Influence of a combination of *Lactobacillus acidophilus* NCFM and lactitol on healthy elderly: intestinal and immune parameters" <u>British Journal of Nutrition</u> 101: 367-375.

33. Sarkar, S. (2010) "Approaches for enhancing the viability of probiotics: A Review" British Food Journal 112: 329-349.

34. Gibson, G.R. and Collins, M.D. (1999) "Concept of balanced colonic microbiota, prebiotics and synbiotics" In: Hanson, L. A. and Yolken, R. H. editors. Probiotics other nutritional factors, and intestinal microflora, 42: 139-152.

35. Zhao, Q., Lee, S.J., Mutukumira, A.N., Maddox, I. and Shu, Q. (2011). "Viability and delivery of immobilized *Lactobacillus reuteri* DPC16 within calcium alginate gel systems during sequential passage through simulated gastrointestinal fluids" Beneficial Microbes 2: 129-138.

36. Hotchkiss, A.T., Liu, L.S., Call, J.E., Cooke, P.h., Luchansky, J.B., and Rastall, R. A. (2002). "Synbiotic Matrices derived from plant oligosaccharides and polysaccharides" In: New Delivery Systems for Controlled Drug Release from Naturally Occurring Materials, ACS Symposium Series 992: 69-77

37. Julien Grimoud, Henri Durand et.al, (2010). "*In vitro* screening of probiotic lactic acid bacteria and prebiotic glucooligosaccharides to select effective synbiotics". Clinical Microbiology 16(5): 493-500

38. Maria Bielecka, Elz, Bieta Biedrzycka, Anna Majkowska, (2002). " Selection of probiotics and prebiotics for synbiotics and confirmation of their in vivo effectiveness" Food Research International 35: 125-131

39. S. Perrin, J.P. Grill and F. Schneider (2000). "Effects of fructo-oligosaccharides and their monomeric components on bile salt resistance in three species of Bifidobacteria" Journal of Applied Microbiology 88: 968-974

40. P. De Boever, R. Wouters and W. Verstraete (2001). "Combined use of *Lactobacillus reuteri* and soygerm powder as food supplement" Letters in Applied Microbiology, 33: 420-424

41. M. Gmeiner, W. Kneifel et.al (2000). "Influence of a synbiotic mixture consisting of *Lactobacillus acidophilus* 74-2 and a fructo-oligosaccharide preparation on the microbial ecology sustained in a simulation of the human intestinal microbial ecosystem (SHIME reactor)" Applied Microbial Biotechnology 53: 219-223

42. T. Jankowski, M. Zielinska and A. Wysakowska (1997). "Encapsulation of lactic acid bacteria with alginate/starch capsules" Biotechnology Techniques 11(1): 31-34

43. T.Y. Sheu and R.T. Marshall (1991) "Improving culture viability in frozen dairy desserts by microencapsulation" Journal of Dairy Science, 74(1): 107-111

44. Wen-Chian Lian, Hung-Chi Hsiao, Cheng-Chun Chou (2002) "Survival of bifidobacteria after spray-drying" International Journal of Food Microbiology, 74(1-2):79-86

45. C. Santivarangkna, B. Higl, P. Foerst (2008) "Protection mechanisms of sugars during different stages of preparation process of dried lactic acid starter cultures" Food Microbiology, 25(3): 429-441

46. J. Burgain, C. Gaiani, M. Linder, J. Scher (2011) "Encapsulation of probiotic living cells: From laboratory scale to industrial applications" Journal of Food Engineering , 104(4): 467-483

47. Hotchkiss A., Olano-Martin, Grace W., Gibson G.R., Rastall R.A. (2003) "Pectic Oligosaccharides as Prebiotics" ACS Symposium Series, ACS 849(5): 54-62

48. V. Patel, G. Saunders, and C. Bucke, (1994) "Production of fructo-oligosaccharides by *Fusarium oxysporum*", Biotechnology Letters, 16(11): 1139-1144

49. Arildo José Braz de Oliveir et.al (2011) "Structure and degree of polymerization of fructooligosaccharides present in roots and leaves of *Stevia rebaudiana* (Bert.) Bertoni" Food Chemistry, 129(2): 305-311

50. Alistair M. Stephen, (1995) "Food polysaccharides and their applications" CRC Press: technology and engineering 9-280.

51. De Man, J. C., Rogosam, Sharpem, E. (1960) "A medium for the cultivation of lactobacilli" Journal of Applied Bacteriology, 23: 130-135.

52. Gibson, A. M., Bratchell, N., Roberts T.A. (1988) "Predicting microbial growth: growth responses of salmonellae in a laboratory medium as affected by pH, sodium chloride and storage temperature" International Journal of Food Microbiology, 6: 155-178

53. Zhao, Z. Y., Liang, L., Fan, X., Yu, Z., Hotchkiss, A. T., Wilk, B. J., Eliaz, I. (2008) "The Role of Modified Citrus Pectin as an Effective Chelator of Lead in Children Hospitalized with Toxic Lead Levels" Alternative Therapies Health Medicine, 14: 34-38.

54. Olano-Martin, E., Gibson G.R., Rastall R.A., (2002) "Comparison of *in vitro* bifidogenic properties of pectins and pectic-oligosaccharides" Journal of Applied Microbiology, 93:505-511.

55. Manderson, K., Pinart, M., Tuohy, K.M., Grace, W. E., Hotchkiss, A.T., Widmer, W., Yadav, M.P., Gibson, G. R., Rastall, R.A. (2005) "In vitro determination of prebiotic properties of oligosaccharides derived from an orange juice manufacturing by-product stream", Applied Environmental Microbiology, 71: 8383-8389.

56. A.L. Ching, C.V. Liew, P.W.S. Heng, L.W. Chan (2008) "Impact of cross-linker on alginate matrix integrity and drug release" International Journal of Pharmaceutics, 355:259–268.

57. Pornsak Sriamornsak, Ross A. Kennedy (2006) "Development of polysaccharide gel-coated pellets for oral administration: Calcium alginate" European Journal of Pharmaceutical Sciences, 29: I39-I47

58. Louis B. Rockland (1960) " Saturated salt solutions for static control of relative humidity between 5ºC and 40ºC" Analytical Chemistry, 32(10): 1375-1376

59. Chalat Santivarangkna, Mathias Aschenbrenner, Ulrich Kulozik, and Petra Foerst, (2011), " Role of glassy state on stabilities of freeze-dried probiotics" Journal of Food Science, 76(8): R151-R156