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Comparative Formulation and Evaluation of Probiotic Products Utilizing different Bacterial Strains: A Comprehensive Study

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ABSTRACT: Probiotic bacterial strains are used in preparations that are good for human and animal health. The advantages of probiotics have led to a rise in interest in methods for microbial preservation. This research contrasts a few probiotic formulation techniques. Here we have considered six bacteria strains *Bacillus subtilis* HFS 2.1 TM, *Bacillus subtilis* HFS 10.2 TM, *Staphylococcus arlettae* FS 9.2 SA, *Pediococcus pentosaceus* HFS 6.2 NA, *Limosilactobacillus fermentum* HFS 11.1 TM, and *Lactiplantibacillus* HFS 11.1 PDA. Three types of probiotics have been formulated capsules by solid-state fermentation, microencapsulated gel beads, and curd. The overall comparison reveals that microencapsulation is the most effective method, followed by solid-state fermentation. The major challenge faced during this study was obtaining a dried soy substrate-grown probiotic formulation, which was successfully achieved by adding additional steps like desiccation, intermediate crushing, and redrying in a hot air oven.

Keywords: Microencapsulation, Solid-state fermentation, Probiotic-curd.

INTRODUCTION

Probiotics are live, nonpathogenic microorganisms that are given to patients to help with microbial balance, especially in the digestive system (Nancy, 2010). Numerous microorganisms found on the skin, in the mouth, and the gastrointestinal tract coexist closely with humans. The GI tract has the highest number of commensal species, some of which are crucial to human health. The development of the gut flora occurs quickly after birth, is largely constant throughout life, and is crucial for maintaining human homeostasis. Alterations in the composition and impact on the flora may result from antibiotics, immunosuppressive therapy, and radiation, among other forms of treatment, dysbiosis brought on by disease, etc. Therefore, reestablishing the microbial balance and preventing disease may be accomplished by introducing advantageous bacterial species into the GI tract (Gupta & Garg 2009). There are several different varieties of probiotic products on the market. Yogurt and kefir, are two probiotic-rich fermented foods that may be found in practically every supermarket However, it is now more common to come across microencapsulated lyophilisates, which are made to release probiotics into the colon, protect them from harsh conditions in the upper gastrointestinal tract, maintain the stability of probiotics during storage, and make it easier for probiotic microorganisms to colonize the mucosal surface. The substances employed in the encapsulating materials are universally acknowledged as safe and suitable for use in the food industry. Additionally, lyophilized probiotics in tablet form are also available, as are chocolate tablets in a variety of shapes (such as gummy bears) or even lollipops (Kiepś & Dembczyński 2022; Zawistowska-Rojek *et al.*, 2022).

Here we have formulated three types of products from each category [A] Fermented curd, [B] microencapsulated beads alginate + CMC beads [C] Capsules from dry powder prepared by solid-state fermentation Sodium alginate is the most typical encapsulating substance because of its simplicity, nontoxicity, biocompatibility, and low cost. Alginates are mucoadhesive; however, they are typically brittle when cross-linked. Alginate microcapsules have reduced drug encapsulation and stability, which can be improved by mixing with appropriate polymers. These limitations can be overcome by combining CMC with alginate. In solid-phase fermentation, the development of advantageous bacteria occurs on a solid substrate, like soy and oil meal, during the manufacturing of probiotic formulations.

Here we have used soybean as the substrate. Six Bacteria were considered for probiotic formulations that are HFS 2.1 TM – *Bacillus sp. OR361754.1*, HFS 10.2 TM – *Bacillus* sp. *OR361756*, FS 9.2 SA – *Staphylococcus arlettae OR361758*, HFS 6.2 NA – *Pediococcus pentosaceus OR361759*, HFS 11.1 TM – *Limosilactobacillus fermentum OR361760* and HFS 11.1 PDA – *Lactiplantibacillus* sp. *OR361761*

Why were these specific bacterial species chosen for Probiotic formulation?

Bacillus sp. – They release enzymes, antioxidants, vitamins, peptides, and antibacterial substances that assist in balancing the gut microbiota and promote

digestion in addition to modulating the host's immunological response. it has shown antiviral activity against adenovirus and avian influenza as well (Colom *et al.* 2021). *B. subtilis* can manufacture antibiotics such as amicoumacin A and nonamicoumacin against *H. pylori* and inhibits the attachment of *Salmonella enteritidis, Listeria monocytogenes,* and *E. coli* to the HT-29 cells (Lee *et al.*, 2019). Reduces oxidative stress, elicits a certain biological reaction, enhances the mood status of the hosts, and does so by activating the gut-brain axis, which causes an increase in 5-HT in the hypothalamus (Jiang *et al.*, 2022). Prevents -synuclein aggregation and dissolves existing clumps and can be used in the treatment of Parkinson's disease (Goya *et al.*, 2020).

Limosilactobacillus fermentum – A probiotic known to have antibacterial and antioxidant properties. Because of its capacity to lower cholesterol levels, *L. fermentum* has been regarded as a probiotic in some circles. Reduces oxidized LDL cholesterol, which is a significant factor in the development of atherosclerosis. The strain modifies the blood's ratio of reduced to oxidized glutathione and raises levels of paraoxonase, an antioxidant enzyme that shields LDL particles from oxidative changes, as two methods that may contribute to the antioxidant effect. Both help avoid oxidative food damage and shield the host from illnesses derived from food (Mikelsaar & Zilmer 2009). In experimental IBS, this probiotic reduced gut inflammation restored altered intestinal permeability, decreased mast cell degranulation, and restored gut dysbiosis. Therefore, our findings point to a potential application of *Limosilactobacillus fermentum* for the treatment of IBS patients in clinical settings (Rodríguez-Sojo *et al.*, 2022).

Pediococcus pentosaceus – Has many probiotic properties, primarily antioxidant, cholesterol-lowering, and immunological effects. It also has probiotic actions against inflammation, cancer, and detoxification (Qi *et al.*, 2021). Might represent a promising candidate, due to the metabolite of GABA, that could be used for the treatment of Parkinson's Disease (Pan *et al.*, 2022).

Lactiplantibacillus sp. – Bacteriocins with intriguing antibacterial activity can be produced by substances with good antioxidant and antimicrobial activities. Reduced levels of inflammatory cytokines and exhibits antifungal qualities (Noemí *et al.*, 2023). Can be used as a probiotic to produce metabolites that alter neuronal pathways, repair the intestinal mucosal barrier, and maintain the balance of the intestinal environment, which can have a direct or indirect anti-anxiety effect. It can help treat anxiety disorders as an adjuvant therapy (Liu *et al.*, 2022).

Staphylococcus arlettae – Nine antibiotic resistance genes, including cfr, erm(C), tet(L), erm(T), aadD, fosD, fexB, aacA-aphD, and erm(B), are encoded by a plasmid that was recently characterised, novel functional *β*-lactamase (bla_{ARL}) was detected as well as additional genes in the genomes relevant to antibiotic resistance, including those for erythromycin (e.g., msrA, mphC), tetracycline (e.g., tetL), and chloramphenicol (e.g., fexA) (Lavecchia *et al.*, 2019). Shown to produce serotonin and GABA which are important neurotransmitters and have many benefits in neurological diseases and disorders.

MATERIAL AND METHODS

A. Solid-state fermentation to obtain powdered probiotics and capsule preparation (Fedorenko et al., 2019).

Preparation of (SSPF) Soy substrate-grown probiotic formulation. After washing, 240 grams of soybeans were soaked overnight. The water was drained after incubation and soybeans were divided into 6 plates 40 gm each for autoclaving after multiple washes. Beans were then cooled down and a 10 mL overnight culture of Probiotic strain in a nutrient medium was added and mixed thoroughly. The inoculated beans were layered in a sterile tray with a non-hermetic lid and incubated for 24 h at 42 °C. The beans were further dried in a desiccator after spreading a layer on sterile filter paper to remove the maximum possible moisture for 3 days. The beans were then dried in a hot air oven at 50°Celcius for 5 days after which they were partially ground in a sterilized mortar and pestle and further incubated in a hot air oven at 50°Celcius for 10 days after this completely dried (SSPF) Soy substrate-grown probiotic formulation was obtained and crushed to a fine powder in mortar and pestle.

Capsule formation. Soy substrate-grown probiotic formulation (SSPF) was placed in a special glass bottle with a well-sealed bung. 20 ml of potassium permanganate solution at 2% was filled in the glass bottle. The SSPF-filled tray was hung above the solution level using a wire and incubated in a hot air oven for 24 hours. The tablets were then put on a clean petri dish and allowed to harden for 24 hours in a hot air oven set to 50°Celcius. Each tablet was prepared using 2 grams of SSPF.

Testing cell viability. For testing cell viability, the tablets were added to 10 ml of nutrient broth incubated overnight at room temperature, and the broth was diluted by serial dilution, 10^{-4} dilutions were plated, and colonies obtained were counted in CFU/ml.

B. Preparation of microencapsulated probiotic beads (Solanki et al., 2016).

In distilled water with 0.75% sodium carboxymethylcellulose and 10^{10} CFU/mL of sample, 3% sodium alginate was added. A sterile syringe was used to inject sodium alginate and the cell suspension mixture into the 5% CaCl2 solution. Gel spheres were instantly produced from the drops. Three centimeters separated the syringe from the $CaCl_2$ solution. The beads were then moved to a sterile screw cap tube, separated by Whatman filter paper filtration, and refrigerated until further usage.

Measurement of Bead size. By using a calibrated ocular stage micrometre to measure the diameters of 20 dried beads at a magnification of about 10, we were able to determine the particle sizes of the prepared beads. Bead mean diameters were computed, and standard deviations were shown.

Calculation of percentage yield. The final product weight after adequate drying was compared to the initial total weight of probiotics and polymers used to make the beads to determine the percentage yield of the prepared batches. The following formula was used to determine the percentage yield:

Percentage yield = Practical mass of beads/Theoretical yield x100

Theoretical yield = wt. of sodium alginate (3%) + wt. of CMC (1.5%) + wt. of $CaCl_2$ (5%)

Calculating probiotic entrapment efficiency (PEE). A sterile conical flask filled with simulated intestinal fluid (pH 6.8) and 0.5g of dried beads was weighed and placed in an orbital shaker to mechanically disintegrate in the simulated intestinal fluid for two hours. 1.0 mL aliquot of the mixture was taken, the necessary dilutions were performed, and the samples were counted for viable colonies using the pour plate method, which is quantified in terms of the number of colony-forming units (CFU). The value of entrapment effectiveness was determined using the formula:

Entrapment efficiency % = $\log_{10}N / \log_{10} N_0 \times 100$

N = Number of colonies formed from beads.

N_0 = Number of free cells added to the biopolymer mixed and number of colonies formed.

Calculating viability in Simulated gastric fluid (SGF). 0.1 g of beads were extracted from each formulation batch and added to 10 mL of simulated gastric fluid to test the viability of the probiotic under in vitro settings (simulated gastric fluid). A 1.0 mL sample was taken after 2 hours of incubation in simulated stomach fluid, and successive dilutions were then performed using anaerobic solution. Using the pour plate method on Nutrient agar, the serial dilutions were exposed to a viable colony count, measured in colony-forming units (CFU). A colony counter was used to count the colonies that formed after the plates were incubated for 24 hours at 37°C.

Observing swelling behavior. The percentage swelling index of the prepared sodium alginate beads was used to evaluate the swelling behavior of the probiotic bacteria included in the beads. A conical flask filled with 200 mL of simulated gastric fluid (pH 1.2) was used to hold beads with a known weight (1 gm) for intervals of 10, 30, 60, 90, and 120 minutes. After each interval, the weight of the beads was measured after being blotted with a piece of filter paper to remove any extra water on the surface and transferred to a fresh medium (simulated gastric fluid) to continue to swell. The beads were put into a conical flask with 200 mL of simulated intestinal fluid after 120 minutes. For a set amount of time (150, 180, and 210 minutes), the weight of the beads was calculated. At each time interval, a new SIF was substituted. On a weighing balance, the mass of the dry beads was established at time zero, and the mass of the wet beads was measured at each sampling point. The following formula was used to calculate the percentage of the swelling index:

Swelling index % = [Weight of beads after swelling-Dry weight of beads]/Dry weight of beads ×100

C. Fermentation of probiotic curd (Yadav et al., 2005).

50ml of raw buffalo milk was purchased, put into stoppered wide-mouth glass jars, and then autoclaved. and without exposing it to the environment, cooled to 37°C. Probiotic bacterial samples were used to inoculate the jars (10^7 CFU/ml) and left at room temperature for further incubation.

Microbiological analysis. We made sequential dilutions of curd samples in sterile normal saline. The number of total bacteria was determined by plating on Nutrient agar and incubating for 72 hours at 37°C. Colonies after incubation were counted.

Physicochemical properties. It was evaluated whether the body and texture were firm or loose and whether the appearance was well-set or not. Additionally, pH was evaluated.

RESULTS AND DISCUSSION

A. Solid-state fermentation to obtain powdered probiotics and capsule preparation

Preparation of (SSPF) Soy substrate grown probiotic formulation. As a Soy substrate-grown probiotic formulation, an entirely dried and finely crushed powder was produced after about 20 days. No spoilage was observed during the fermentation or drying process. The entire procedure was performed while maintaining a sterile environment.

Fig. 1. SSPF formulation.

Capsule formation (Fig. 2). The capsules were well-formed and weighed approximately 2 grams after hardening and a complete loss of moisture.

Fig. 2. Capsule formation.

Testing cell viability. Cell viability assessment ensures the survival of the probiotic bacteria in the formulation. Good cell viability was obtained in all six probiotic bacterial capsules; maximum viability was obtained for sample HFS 2.1 TM. 282 CFU/ml followed by HFS

10.2 TM – 276 CFU/ml, HFS 6.2 NA – 266 CFU/ml, HFS 11.1 PDA – 257 CFU/ml, HFS 11.1 TM – 251 CFU/ml and FS 9.2 SA – 248 CFU/ml.

B. Preparation of microencapsulated probiotic beads

Fig. 3. Microencapsulated probiotic beads.

Measurement of Bead size. The average bead size obtained were, HFS 2.1 TM- 24.35mm, HFS 10.2 TM- 26.05mm, FS 9.2 SA- 18.05mm, HFS 6.2 NA- 21.05mm, HFS 11.1 TM- 20.15mm and HFS 11.1 PDA- 18.25mm.

Calculation of percentage yield. Percentage yield indicated the amount of beads formed from the total substrate ($CaCl_2$, CMC, and alginate) used. A satisfactory percentage yield was obtained, ranging from 78.95% to 94.5%. The highest percentage yield was obtained for sample HFS 10.2 TM.

Table 1: Percentage yield of microencapsulated probiotic beads.

Sample code	Practical mass of beads	Theoretical yield	Percentage yield
HFS 2.1 TM	16.2 gm	17.5 gm	92.6%
HFS 10.2 TM	16.53 gm	17.5 gm	94.5%
HFS 6.2 NA	16 gm	17.5 gm	91.43%
FS 9.2 SA	13.816 gm	17.5 gm	78.95%
HFS 11.1 TM	15.09 gm	17.5 gm	86.2%
HFS 11.1 PDA	14.611 gm	17.5 gm	83.5%

Calculating probiotic entrapment efficiency (PEE). Probiotic Entrapment Efficiency indicates the amount of probiotic bacteria entrapped within the microcapsules. Maximum PEE was obtained for sample FS 9.2 SA.

Table 2: Probiotic entrapment efficiency of microencapsulated probiotic beads.

Sample code	N_o	N	PEE
HFS 2.1 TM	8.77	8.54	97.38%
HFS 10.2 TM	8.33	7.97	95.67%
HFS 6.2 NA	8.62	8.27	95.94%
FS 9.2 SA	8.27	8.13	98.31%
HFS 11.1 TM	8.13	7.91	95.30%
HFS 11.1 PDA	8.59	8.33	96.97%

Calculating viability in Simulated gastric fluid (SGF). In SGF, cells had good viability ranging from 7.97 Log CFU/ml to 6.59 Log CFU/ml, with HFS 2.1 having the maximum viability of 7.97 log CFU/ml followed by HFS 11.1 TM - 7.91 log CFU/ml, HFS 11.1 PDA - 7.63 log CFU/ml, HFS 6.2 NA - 7.22 log CFU/ml, FS 9.2 SA - 7.1 log CFU/ml and HFS 10.2 TM - 6.59 log CFU/ml.

Observing swelling behavior. It is desirable for probiotics to have microencapsulated beads swell somewhat in the stomach and slightly more in the intestine since this increases the release of bacteria.

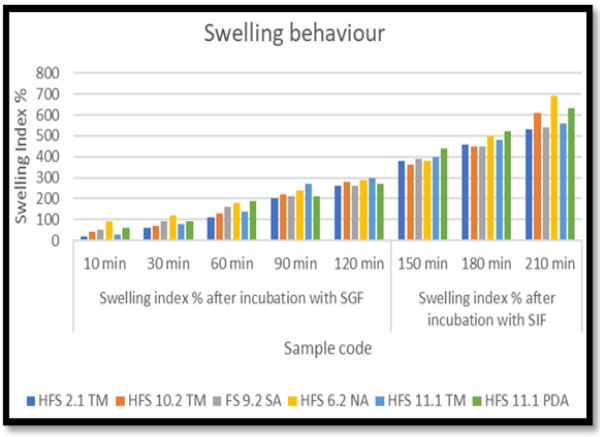

Fig. 4. Swelling behavior of microencapsulated probiotic beads.

C. Fermentation of probiotic curd (IS 1479-1 1960)

Microbiological analysis. Bacterial counts gradually increased from 24 to 72 hours, mostly ranging from 7.14 Log CFU/g to 8.97 CFU/g. The highest counts were obtained for HFS 6.2 NA.

Physicochemical properties. Appearance: After 24 hours of incubation, isolates HFS 6.2 NA and HFS 11.1 TM produced firm and well-set curd, followed by isolate HFS 11.1 PDA after 48 hours. pH: The survival and growth of the integrated probiotic are intimately correlated with the pH of the food product (Vivek *et al.*, 2022). pH initially reached 6 and then fell to 5 after 72 hours of incubation. At the same time, isolates FS 9.2 SA, HFS 10.2 TM and HFS 2.1 TM produced curd that had a loose body, a texture that did not seem well-set, and a pH that dropped from 7 after 24 hours to 6 after 72 hours.

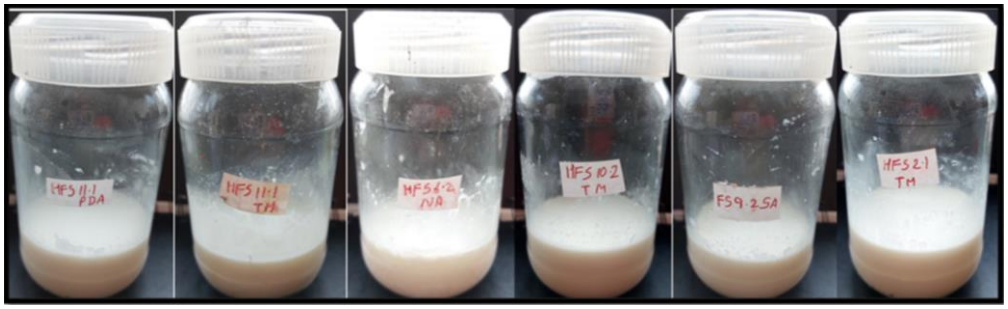

Fig. 5. Probiotic curd.

CONCLUSIONS

The best method for probiotic formulation is solid-state fermentation because it preserves stability, and viability without sacrificing shelf life due to its dry state. The only drawback of this method is spoilage during SSPF fermentation and the growth of undesirable bacteria which can be prevented by maintaining a sterile environment. Microencapsulation is the second best method for probiotic formulation as it also serves good survival and stability but the primary drawback to this procedure is the lower shelf life and survival rate of bacteria, due to its shorter shelf life, susceptibility to spoilage, and inability to be produced by non-fermenting bacteria, Also dairy products raise the risk of lactose intolerance, galactosemia, milk protein allergies, and excessive cholesterol in humans. Thus, curd formation appears to be the least practicable method. All six bacteria can be used to successfully create capsules using solid-state fermentation, as well as microencapsulated beads, but bacteria that do not ferment cannot be utilised to create probiotic curd.

FUTURE SCOPE

The promising hybrids identified in this study can be exploited commercially to cure human illnesses. The study provides a scope for formulating potent probiotics for dedicated illnesses. It also offers customizable options and promotes designer drug culture by designing probiotics specific to patient's requirements. The comparison between probiotic techniques provides a surplus benefit of choosing between the right form of probiotic which can be used to treat the conditions.

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Conflict of interest. None.

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