Study of Probiotics Viability and Attributes Stability in Powdered Milk Prepared by Lyophilization

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In

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In the name of Allah, the Most Merciful, the Most Kind

DEDICATION

I dedicate my work to my parents, brothers, and friends who have helped and guided me in every aspect of life. It would not have been possible without their love and support. Plus, this research is dedicated to all the individuals who are passionate and determined to explore the unseen with the desire to bring about great revolution in the field of science and technology.

DECLARATION

The information and content contained in this thesis is my original work. I have not introduced any piece of this work somewhere else for any degree.

Alviya Zainab Kazmi

CERTIFICATE

This thesis, submitted by Alviya Zainab Kazmi to the Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of **MPhil Microbiology.**

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LIST OF ACRONYMS

A	Absorbance
AIDS	Acquired Immune Deficiency Syndrome
BSH	Bile Salt Hydrolase
$CaCl_2$	Calcium Chloride
e.g.	For example,
WHO	World Health Organization
TSA	Tryptic Soy Agar
OGA	Oxytetracycline Glucose Agar
FTIR	Fourier Transform Infrared Spectroscopy
HIV	Human Immunodeficiency Virus
HMOs	Human Milk Oligosaccharides
ATCC	American Type Culture Collection
i.e.	That is
LAB	Lactic Acid Bacteria
mg	Milligram
MHA	Mueller-Hinton Agar
ml	Milliliter
MRSA	De Man Rogosa and Sharpe Agar
NaCl	Sodium Chloride
nm	Nanometer
OD	Optical Density
CMA	Cow Milk Allergy

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All Praise be to Almighty ALLAH, the Omnipotent, the most Compassionate, Peace and greetings be upon the Holy Prophet Muhammad (PBUH) and his descendants (AS), the most immaculate among every person at any point conceived on the Earth's surface, who is everlastingly a wellspring of direction and information for mankind.

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Alviya Zainab Kazmi

ABSTRACT

Synthetic infant formulas have served the purpose of nutrition, but they come along with food intolerance and food allergy. To come up with this issue, milk from five different sources i.e. Cow milk, goat milk, sheep milk, soy milk, and skim milk were fermented using characterized *Streptococcus thermophilus* QAUSTN63, *Lactobacillus delbrueckii subsp. bulgaricus* QAULDN14, and *Lactococcus lactis* QAULLNA8, and *Geotrichum candidum* QAUGC01 (PRJNA523005) followed by lyophilization. The proximate analysis of the milk products was carried out before (per 100ml) and after lyophilization (per 100g) to ensure nutritional intactness. The results showed highest crude protein in soy milk i.e. 6.0% and 27.01%, highest ash content i.e. 1.053% and 5.48%, and fat content i.e. 8.5% and 29.07%, total solids 19.44% and 96.82% in sheep milk in liquid and lyophilized product respectively. Alongside this, the probiotic attributes stability was carried out for bacterial consortium and *Geotrichum candidum* isolated from each lyophilized milk samples. All the cultures were found to be viable up to 10^8 CFUs/ml. Lastly, the probiotic attribute stability tests showed that both the bacterial consortium and *Geotrichum candidum* gave positive Bile salt Hydrolase (BSH) activity, proteolytic activity, lipolytic activity, NaCl tolerance, and bile salts tolerance. Whereas, lactose fermentation, milk fermentation, and antibiotic susceptibility were positive only for bacterial consortium. The antimicrobial activity against *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 14028, *E. coli* ATCC 25922, *Bacillus subtilis* ATCC 5230, and *Staphylococcus aureus* ATCC 25923 was observed for bacterial consortium, while *Geotrichum candidum* was antifungal susceptible with positive antimicrobial activity against *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, and *Staphylococcus aureus* ATCC 6538 along with the aforementioned strains. The optimum temperature for bacterial consortium and *Geotrichum candidum* was 37ºC and 25ºC, along with 6-8 and 4-6 as optimum pH for both respectively. All these tests proved that the strains were active after lyophilization with all the milk nutrients intact in each product. Hence, the present study was believed to be the most reliable natural fermented alternative to synthetic infant formulas.

CHAPTER 01
INTRODUCTION

INTRODUCTION

Microbiology existed even before the advent of the solar system. The universe is familiar with microorganisms more than any other existing species. And therefore, it has made microbiology and its wonders an essential part of its existence. Owing to this fact, the role of microbiology in the life of humans, Crown of Creation, is undeniable. Microorganisms make an essential part of every human before and soon after the birth. When talking about the human body, the most important part is the gut. Because of its cruciality in the human body, nature has selected it as the main house for microorganism called as the "gut microbiota". From the time a little human takes birth to the day he enters adulthood, microorganisms play a pivotal role in all biological activities occurring inside human body. Also, among the gut microbiota, probiotics have been declared as the matchless species with profound benefits and amazing properties. For this purpose, nature also chose to load breast milk, the first ever diet of an infant, with a variety of probiotics that help in developing the body throughout life. Breast milk is a rich source of proteins, carbohydrates, lipids, minerals and vitamins. Along with these contents, bioactive agents form a major portion of its composition. These include the probiotics that not only work for immune system maturation and protection against infections, but later on become an essentiality for human gut (Lyons et al., 2020). The most commonly found bacterial species in healthy human milk are *Staphylococcus* including *S. epidermidis, S. hominis, S. capitis and S. aureus; Enterococcus* including *E. faecium* and *E. faecalis*; *Streptococcus* including *S. salivarius, S. mitis, S. parasanguis* and *S. peores; Lactobacillus* including *L. gasseri, L. rhamnosus, L. acidophilus, L. plantarum, L. fermentum, L. salivarius* and *L. reuteri; Lactococcus spp, Bacteriodes spp, Bifidobacterium spp* and *Clostridium cluster* (Walker & Iyengar, 2015). These microorganisms stabilize the gut by providing benefits like antimicrobial effects, immunomodulatory properties and gastrointestinal benefits. In other words, breast milk not only serves as a source of probiotics, but as a support of probiotics too (Lyons et al., 2020). However, over the course of time, there has been a drastic change in the feeding practices. Apart from the apathetic mindset, some medical issues of both mother and infant have compelled industries to look for the alternative to breast milk. HIV and AIDS positive mothers are not advised to breastfeed in order to prevent viral transmission to the baby, mothers on any antimicrobial medication for severe infections or the mothers that are addicted to excessive

drug intake are forbidden to feed the child so that he may be protected from its adversities, mothers with cancer therapies are advised to avoid breast feeding as cancer chemotherapeutics may hinder cell division in infant too, mothers who are undergoing radiation treatment of the chest are also forbidden to breastfeed until their therapy sessions, untreated tuberculosis may also be one of the reasons to stop breastfeeding an infant, mothers positive with the infection human T-cell lymphotropic virus type I or type II should avoid breastfeeding their babies and lastly, the infants who are galactosemic (a condition of lactose and galactose intolerance) are not fed breastmilk rather are prescribed lactose and galactose free formula diet. These motives levelled the grounds for bringing formula feeding in practice(Organization, 2017). Nevertheless, the development of formula milk was just an attempt to mimic breast milk, but the manufactured products have never been able to provide as much advantages as breast milk to date. A few years later, it was found that the formula-fed infants face a lot of medical problems later in life. A comparison of the gut microbiota of exclusivelybreast-fed and exclusively formula-fed infants showed that the latter have a greater diversity of *Streptococcus, Enterococcus, Lachnospiraceae, Veillonella and Clostridioides.* These changes in the gut microbiota are the main reasons to pose long-term health risks to the infants. Some of the most common and prevalent disorders include obesity, atopic diseases, and chronicinflammatory diseases (Ma et al., 2020). At this moment, it has become very crucial to look for a workable solution to this problem. One of the most reliable solution is the replacement offormula milk with easily available animal-source milk i.e. cow milk, goat milk, and sheep milk.Nonetheless, many people still believe that milk from animal sources is indigestible and can cause gut-related issues in infants. Additionally, the fats in animal-source milk have been considered very heavy to be digested by delicate infant guts. This is because the new born infant's exocrine pancreas is immature, and secretes very low quantities of lipase enzyme. Torule out this problem skim milk-based formula diets can be a beneficial replacement to animal-source milk. Above all, lactose intolerance has become quite common in the past few years. Any kind of milk, be it skim or from an animal source, contains lactose as the main carbohydrate moiety. Due to this reason, use of these types of milk becomes impossible for galactosemic or lactose-intolerant infants. But, the scientific world did not turn its back to thisissue and came up with soy milk and soy milk-based infant formula diets (Verduci et al., 2020).However, use of the aforementioned formula milk types may pose a health problem for new born infants. Plus, the shelf-life of animal-

source milk and soy milk is very less making them insufficient for regular use. So, another strategy could be extension in the shelf-life of these milk types with extended benefits for infant health. And, to subside these problems, fermentedinfant formulas have come into play. Fermentation has long been known as the process to improve nutritional composition of a food substance along with increasing its shelf life. Hence,it would not be wrong to say that fermentation is a preservation technique too. The trend of using fermented infant formulas has increased because mothers are now aware of the advantages they provide in the form of nutrition and health safety. The use of probiotics, especially Lactic Acid Bacteria (LAB) for carrying out the fermentation of milk has been a historical practice. Surprisingly, this method is still in use today and is favorable in many ways. For instance, fermented foods have been reported to possess hypocholesterolemic, antioxidant,hypotensive, and anti-allergic effects. Not only this, but they are best known for providing immunological benefits, such as effects on the host's intestine and microbiome, anti-carcinogenic, and immunomodulation properties (García-Burgos et al., 2020). Studies have revealed that the use of LAB fermented formula milk aids in immune system maturation, maintenance of microbiota, and development of metabolome (Roggero et al., 2020). In addition, yeast-fermented formula milk products are also available and used widely as two different microbial cultures improve the fermentation process. However, it may be noted that the microbial cultures being used do not possess antagonistic effects against one another. Plus,yeasts and fungi do not possess the ability to ferment lactose, and therefore they are used as secondary cultures in fermentation processes. LAB such as *Streptococcus thermophilus, Lactobacillus delbrueckii,* and *Lactococcus lactis* have been used widely in industrial preparation of yogurt and cheese. Owing to the health benefits provided by yogurt and cheesedue to these species, the next step is to use them for the preparation of fermented formula milk.These species have been reported to provide cholesterol lowering effects, tolerance to bile salts, lipolysis, proteolysis, antimicrobial effects, and immunomodulation. No doubt, the benefits ofLAB in fermentation of milk are unavoidable but the final acidity and alteration in nutritional composition of milk is a matter of concern (Sharma et al., 2023). To combat this, use of safe edible yeasts such as *Geotrichum candidum* as a secondary culture is a good choice (Šipošováet al., 2021). The fermented milk products may have an extended shelf life as compared to rawand even heat treated but they cannot stay for as long as months or years. Therefore, a long- term storage and preservation technique is needed. Additionally, the

viability of different probiotic strains and nutritional composition of the fermented milk throughout the shelf life isa challenge for many researchers. The viability and stability of probiotic bacteria and yeast in fermented milks depends on different factors, such as the strains used, interaction between microbial cultures, culture conditions, chemical composition of milk, final pH, growth promoters and inhibitors, dissolved oxygen, level of inoculation, and storage temperature (Tamime et al.). Nevertheless, this has not been a problem anymore since the introduction of lyophilization has not only promised viability of the strains but also intactness of the nutritional contents of the milk. So far, lyophilization or freeze-drying has been the most suitable technique to preserve food products especially dairy and fermented foods. Still, lyophilization comes with a risk of alteration in attributes of the inoculated strains and ingredients of the food product (Guiné, 2018).

Combining all the aforementioned phenomena, a logical solution to solve infant nutrition problems can be the development of lyophilized fermented animal-source (cow, goat, and sheep), skim, and soy milk products as infant formula diet. As a matter of fact, formula milk could never be a substitute for breast milk if compared on the basis of microbiology, for a totalof 600 bacterial species have been reported in breast milk and only a few species in formula milk (Witkowska-Zimny & Kaminska-El-Hassan, 2017). Yet, the initiative has been taken withthe hope that the products will somehow improve the gut health of infants deprived of breast milk.

AIM AND OBJECTIVES

Aim

Preparation of LABs (*Streptococcus thermophilus* QAUSTN63, *Lactobacillus delbrueckii* subsp. *bulgaricus* QAULDN14, *Lactococcus lactis* QAULLNA8) and yeast (*Geotrichum candidum* QAUGC01) fermented lyophilized infant formula milk products

Objectives

- To develop nutritionally rich and safe alternatives of infant formula milk by fermentation
- To evaluate the viability and attributes stability of probiotics in the final products

CHAPTER 02
LITERATURE REVIEW

LITERATURE REVIEW

Nature has always been very just when distributing nutrition according to every creation's need. What sunlight is to plants, grass is to herbivores, and grains are to birds, is what breast milk is to infants. A comprehensive definition of breast milk can be the "whole nutrition" for a human being for some early period of life. From the beginning till date, mother's milk has been known as the unrivaled source of nutrition and the only body fluid for ensuring optimal infant growth and development. Apart from providing nutrition, it improves the function of Gastrointestinal tract, immune system and brain, and lessens the programming of late metabolic diseases such as obesity and type II diabetes (Savino et al., 2013). In other words, breast milk provides all required nutrients, medicinal properties, immunomodulation, and health benefits. Therefore, The World Health Organization (WHO) recommends breastfeeding for the first six months of life (Organization, 2017), the American Academy of Pediatrics states the breastfeeding duration to be at least twelve months (Breastfeeding et al., 2012), and Academy of Nutrition and Dietetics affirms that the infant may rely entirely on breast milk up to six months of age and continued to be breastfed with other complementary foods till twelve months of age (Lessen & Kavanagh, 2015). The reason that breastfeeding needs to be continued for a specific time duration is that the contents of breast milk enter in the form of set doses in infant's body. The main constituents of breast milk include carbohydrates, proteins, fats, vitamins, minerals, digestive enzymes and hormones. In addition to these nutrients, it is rich in immune cells i.e. macrophages, stem cells, and other bioactive molecules. Some of these bioactive molecules are protein-derived and lipid-derived, while others are protein-derived and indigestible, such as oligosaccharides, also called as Human Milk Oligosaccharides (HMOs). Alongside this, over 600 bacterial species along with some yeast species have been reported in breast milk that add to its microbiological significance even more. These microbes are the main colonizers of infant gut and support the bodily activities throughout life (Boix-Amorós et al., 2019).

The choice of breastfeeding is entirely personal and is also influenced by certain factors that interrupt breastfeeding practices. Worldwide, 38% of the infants are breastfed exclusively, while in the United States, only 75% of infants are breastfed soon after the birth. However, by the age of 3 months, 67%, or 2.7 million, of infants utilize infant formula for some part of their nutrition. 43% of the new mothers in the United States breastfeed their children up to 6 months of age with

only 13% breastfeeding their young ones exclusively for six months (Chung et al., 2008). In conditions where breastfeeding could not be continued, infant formula or animal milk serves the purpose. Although the use of infant formula as a substitute for breastmilk is not reliable, but every possible effort has been made to try to mimic the nutritional profile of the breast milk (Callahan et al., 2022).

2.1. Animal Milk is Indigestible by Infants, Myth or Fact?

It has long been believed that animal milk is not good for infant health as it can cause serious health issues. In fact, it is still believed that animal milk is way too heavy to be digested for the delicate infant guts. However, the health issues that have been reported in the past and today due to the consumption of animal milk have another hidden side. Exploring the real cause of the health issues raised after feeding an infant with animal milk has uncovered many hidden facts that a few people know about. In general, mothers still prefer artificially synthesized formula milk rather than opting for natural milk sources.

2.1.1. Cow Milk Allergy (CMA) in Infants

Food allergy has always been a common concern of the public health sector due to its increasing rate and prevalence. Food allergies occur as a result of immunological reaction to certain foods affecting the body organs in return. CMA is reported to have been observed in 2.5% infants under 2 years of age and occurring with 12–30% prevalence in infants less than three months old (Fiocchi et al., 2016). The infants facing CMA could either have IgE-mediated atopic mechanisms or T cell-mediated reactions. Moreover, the infants either fed on cow milk or breast milk of the mother using cow milk in her diet, can develop CMA (Manuyakorn & Tanpowpong, 2019). The main allergen in cow milk is the whey protein Beta-lactoglobulin, absent in breast milk. Moreover, it has been reported that proteins casein, Beta-lactoglobulin, and alpha-lactalbumin are the common allergens in cow milk. The allergic reactions include urticaria, angioedema, atopic dermatitis, infantile colic, gastro-oesophageal reflux, oesophagitis, infantile proctocolitis, food-associated enterocolitis, and constipation (Prasad & Shivay, 2020).

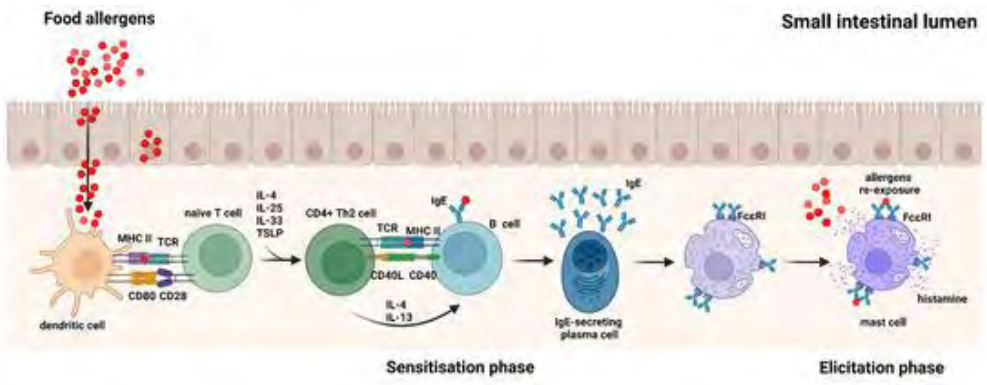


Figure 1 Mechanisms of IgE-mediated food allergy in two phases i.e. Sensitisation phase and Elicitation phase (Maryniak et al., 2022)

However, the exact mechanism of CMA is still unknown. Many believe that it is nothing else but food intolerance that can be cured by the use of hypoallergenic formulae, hydrolyzed formulae, amino-acid formulae, and avoidance of cow milk in breast feeding mothers. As mentioned earlier, the occurrence of CMA is very rare, and that cow milk is a safe food for most of the infants (Arasi, Cafarotti, & Fiocchi, 2022).

2.1.2. Goat Milk Allergy in Infants

Goat milk has got remarkable characteristics that make it a unique food. It has been used as a successful alternative to cow milk after the discovery of CMA. Plus, goat milk proteins genetically differ from cow milk proteins, and goat milk fat has a much better profile than that of the cow milk. Over 40 to 100% of infants allergic to cow milk can tolerate goat milk. In short, goat milk has been known well for its hypoallergenic properties. In addition, goat milk having αs1-casein, a protein polymorph present in cow milk as well, has more allergenicity than that of the goat milk with αs2-casein. Plus, the content of αs1-casein in goat milk is very less and this supports lesser curd yield, longer rennet coagulation time, more heat lability, and weaker curd firmness (Sonu & Basavaprabhu, 2020). Due to these factors, goat milk is much more digestible in the human gut. Studies have also revealed that the consumption of goat milk improves utilization of fat and weight gain in infants with reduced cholesterol and LDL levels. Also, the levels of triglyceride, HDL, and GPT remained normal in infants adding to goat milk benefits. Hence, the concept of goat milk allergy and indigestibility is nothing but a myth that has been ruled out by the scientific and experimental research (Anghel et al.).

2.1.3. Sheep Milk Allergy in Infants

Sheep milk has been known for its ability to provide high energy, fat content, and proteins to the consumers. According to the reports, infants allergic to cow milk can also develop allergy against

sheep milk because of the presence of milk proteins that initiate positive cross- immunological reactions (Nayak, Ramachandra, & Kumar, 2020). Sheep milk has been rendered as a good alternative of cow milk as it has high amino acid sequence identity with counterpart cow milk proteins. It contains a different profile of the specific proteins, with a higher amount of β- and αs2-casein and lower amount of κ- and αs1-casein than cow milk. Currently, no sheep milk allergies have been reported in the Allergen Nomenclature (Sudharson et al., 2021). Nevertheless, many individuals with no CMA history have been reported to develop allergy after eating sheep milk cheese but the two have not been linked yet. In short, sheep milk has more of goat milk like characteristics that are indicative of its safe and non-allergenic profile. Hence, the concept of sheep milk allergy is another myth that has stopped mothers from using it as an alternative to breast milk.

2.2. Mammalian Milk Proteins-based Infant Formula

During the preindustrial period, the infant survival was dependent on mother's milk or its substitution by wetnurse's milk. In case of unavailability of both, infants were fed with animal milk, pre-chewed foods or paps that were poor in nutrients and contaminated causing high mortality rates (Stevens, 2009). By this time, the infant formula milk took birth. The first commercial infant formula was developed by a German chemist, Leibig, in 1867 and rapidly gained popularity in Europe. The product did not challenge breast milk rather justified itself as an attempt to develop a feed identical to breast milk nutritionally. It consisted of wheat flour, malt and potassium bicarbonate, and was to be mixed with preheated milk to form the fully nutritional feed for the infant (Wei, 2019). However, 1874 brought another infant formula product that stated to be the best alternative for feeding children, as it contained powdered milk, wheat flour, malt and sugar, and required water for mixing rather than pre-heated milk. Though it was widely available in Europe and United States, the high price rendered it unaffordable for many (Schuman, 2003). These events paved a way for research in development of a finest substitute for breastmilk. The utmost goal was to formulate a humanized product that matches the nutritional profiling of the breastmilk to its maximum. Emphasis was put on the proportions of protein, fat and carbohydrates rather than on the energy provided. With recognition of calorie requirements, a daily intake of 100 kcal/kg during the first months of life was recommended (Castilho, 2010). However, the synthetic infant formulae were not sufficient enough in termsof nutrition. Contrary to them, infant formulae based on mammal milk proteins provide a much broader range of nutritional energy and benefits.

Due to its greater availability, cow milk is the main protein source in manufacturing infant formula milk (Roy et al., 2020). Nonetheless, due to its allergenicity, the scientific world and industries have shifted to the idea of manufacturing other mammalian protein-based formulae such as goat, sheep, and camel etc. Presently, cow and goat milk proteins are being used in Europe as per the EU legislation. In general, *Capra hircus* (goat), *Ovis aries* (sheep), and *Camelus dromedaries* (camel), contributes approximately 17% of the global milk production. It may be noted that the composition of mammalian milk is different to that of breast milk in terms of total protein content, casein-to-whey protein ratio, composition of the proteins, and the differences in amino acid sequences (Faye & Konuspayeva, 2012).

2.2.1. Cow Milk-based Infant Formula

Cow milk production accounts for a total of 81% worldwide. Due to its free availability, most infant formulas are cow milk-based. Cow milk-based formulas are prepared by reconstitution of milk, where different milk fractions, i.e. proteins (whey or caseins), fats, micronutrients, macronutrients, and non-milk-based ingredients, are mixed together to attain a full diet fulfilling the nutritional needs of infants (Roy et al., 2020). Both whey and casein based infant formula are manufactured and used. Nonetheless, the production and utilization of whey protein formulas has increased after the discovery of its optimal utilization in cheese production (Fenelon et al., 2019). Owing to the problem of CMA, cow milk-based formulas are not recommended for allergic infants. Still, enzyme hydrolyzed cow milk-based infant formulas are now available to solve the issue of CMA.

2.2.2. Goat Milk-based Infant Formula

Goat milk is widely available and used as a common food source in Europe, Asia, Australia, and New Zealand. It is generally used in the production of yogurt and cheese, but is now an ingredient for the production of infant formula as well. It acts as a source of proteins, micronutrients, and macronutrients (Vandenplas et al., 2021). The amount of proteins in goat milk are comparable to that in the cow milk with former having a higher ratio of caseins to whey protein i.e. 84:16 in goat milk and 80:20 in cow milk. The individual protein profile differs with lower quantities of αs1-casein and a higher proportion of αs2-, β-, and κ-casein in comparison with the cow milk. Plus, the specific whey proteins area comparable to that in cow milk (Ceballos et al., 2009). The European Food Safety Authority (EFSA) declared goat milk as a suitable source of proteins for infant formula in the year 2012 (EFSA Panel on Dietetic Products & Allergies, 2012). For long, it has

been believed that goat milk formulas are a good alternative for cow milk formulas for infants with CMA. However, it has been found out later that individuals with CMA can also manifest cross-reactivity to goat milk proteins. In some cases, individuals showed goat milk allergy with no evidence of CMA (Hazebrouck et al., 2014). Nevertheless, the specificity of IgE-binding capacity does not allow cross reactivity even with a difference of 2-3 amino acids in the polymorph of a single protein e.g. β-casein of both cow and goat milk. Hence, the allergenicity of goat milk proteins is by far a vague concept with not even a single protein reported as allergen yet (Bernard et al., 2012).

2.2.3. Sheep Milk-based Infant Formula

When talking about sheep milk, it is available widely in China, New Zealand, Turkey, Greece, Syria, and Romania. The proteins in sheep milk are comparable to that of cow milk with a similar casein to whey protein ratio i.e. 80:20. The profile of specific proteins is different with a higher amount of β- and αs2-casein, and lower amount of κ- and αs1-casein than cow milk (Balthazar et al., 2017). The concept of sheep milk formula is not very common around the globe. Plus, it has not been approved in Europe due to which it is currently unavailable in China and New Zealand. It is suspected that sheep milk proteins may cause allergic reactions in infants due to protein homology with cow milk proteins. But, none of the sheep milk proteins has been declared as an allergen to date (Viñas et al., 2014).

2.3. Plant-based Infant Formula Milk

Over the course of years, the idea of infant formula production using mammalian milk proteins has been substituted by a much sustainable and environment friendly alternative- plant proteins-based infant formulas. This notion has provided a solution to CMA, lactose intolerance, vegan habits, taste preferences, and ethical issues. In other words, plant proteins in infant formula milk is the most eco-friendly dietary solution presented so far (Martini et al., 2021). The plant-based beverages can be divided into five categories viz. cereal-based (oat, rice, corn, spelt), legumes-based (soy, peanut, lupin, cowpea, chickpea), nut-based (almond, coconut, cashew, hazelnut, Brazil nut, pistachio), seed-based (sunflower, sesame, hemp), and pseudocereal-based (quinoa, teff, amaranth) beverages (Silva, Silva, & Ribeiro, 2020). Manufacturers have now started making plant-based infant formulas but the products do not necessarily satisfy the nutritional requirements of the infants. Moreover, not all plant proteins provide as much benefits as mammalian milk proteins. As far as the advantages are concerned, soy-based and hydrolyzed rice-based infant

formulas are manufactured and used for infants (Vitoria, 2017).

2.3.1. Soy-based Infant Formulas

Soy bean is a legume crop that is known for its high-quality protein content making upto 40% of the dry weight. Soy bean-based infant formulas are manufactured and sold in many countries around the entire, with North America being the hub. When talking about infants with CMA, soy-based formulas are tolerated well by 90% of the allergic infants (Verduci et al., 2020). Soy- based infant formulas have taken over the market for more than a century now, although they have been changed in many respects throughout this time. At first, the soy-based infant formulas were based on soy flour to soy protein isolates to obtain a higher digestibility and a lower content of fibres and phytates (Vandenplas et al., 2014). Later on, fortification was done by addition of the amino acids viz. methionine, taurine, carnitine, choline and inositol. Presently, the soy-based infant formulas have been supplemented with LCPUFAs (Westmark, 2017). Despite the initiatives to improve soy-based infant formulas, concerns have been raised regarding potential risks due to the phytate and phytoestrogen content as well as nutritional deficiencies (Vandenplas et al., 2011). Yet, meta-analysis, has revealed that soy-based infant formulas are a safe alternative to cow's milk-based infant formulas (Vandenplas et al., 2014). In addition, soy allergy affects only 0.3-0.4% of young children only which is much less than the lot affected by CMA. Also, soy does not contain cow milk proteins and lactose, and therefore, it is a good alternative for infants facing food allergy and food intolerance (Verduci et al., 2019). The cross-reactivity of soy proteins and cow milk proteins is not common making soy's profile even more reliable. However, it may be noted that soy-based infant formulas must be avoided in high-risk infants (Kattan, Cocco, & Järvinen, 2011).

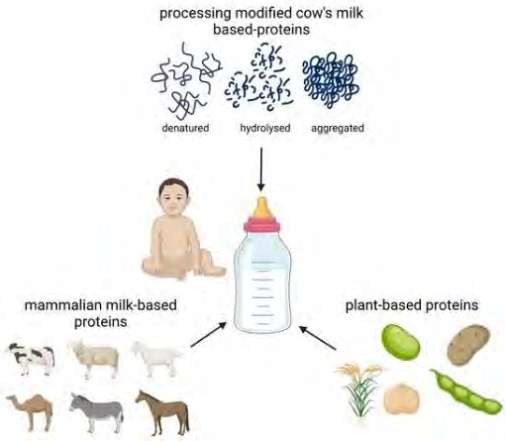


Figure 2 Types of proteins used for infant formula manufacture (Maryniak et al., 2022)

2.4. Probiotic Supplementation in Infant Formula

The nutritional profile of breast milk can be mimicked by combining all the ingredients in the form of formula milk, or using mammalian milk-based and plant protein-based formulas. But, the microbiological profile of breast milk is far beyond the scope of science to be replicated in some artificially synthesized or modified product. As mentioned earlier, human breast milk contains up to 600 species including *Staphylococcus, Enterococcus, Streptococcus*, *Lactococcus spp, Bacteriodes spp, Bifidobacterium spp* and *Clostridium cluster*. Along with the nutritional compatibility of the formula milk with breast milk, supportive microbiology is also essential. In short, the supplementation of formula milk with probiotics showed a more dependable way of manufacturing infant formula (Musilova, 2014). So, a better way to cope with the problem is the supplementation of formula milk with probiotic bacterial or fungal strains. The probiotic strains added to the formula milk can be isolated either from food or from human fecal samples. Among the probiotic strains, *Bifidobacteria and Lactobacilli* have gained much focus due to potential health benefits. The breastfed infant's gut is dominated by both these species so the formula milk companies have shifted towards adding these in their milk products to make the gut of formula fed infants comparable to the gut of breastfed infants (Walker & Iyengar, 2015). It may be noted that it is humanly not possible to add all the known bacterial and fungal species from breast milk into formula milk. Therefore, only a few potentially beneficial strains are selected to extend the nutritional and microbiological value of the probiotic supplemented formulas. On the other hand, it is not necessary to introduce strains that are present in breast milk only, rather strains from other edibles, such as yogurt and cheese, can also be considered (Gao et al., 2021).

2.4.1. Benefits of Probiotic Supplementation in Infant Formulas

The beauty of using probiotic strains lies not only in the fact that it is good for gut health, but also in the reality that the introduced microbiota acts as a means to reduce allergenicity of the raw material. For instance, after heat treatment of the raw milk, the introduced probiotic strains can tend to ferment the milk making it nutritionally more valuable, increasing its shelf life, and modifying the allergens such as milk proteins (Golkar, Milani, & Vasiljevic, 2019).

Process modifications such as exposure to radiation, high pressure, heat treatment, enzymatic hydrolysis, and fermentation possess the ability to reduce or destroy IgE-binding epitopes by destroying, denaturing, and degrading the milk proteins. Instead of carrying out the process

chemically or mechanically, the best and the most cost-effective way is by the introduction of probiotic bacteria and fungi. Not only will they improve the nutritional value and promote gut maturation, but will act as harmless agents of the aforementioned process modifications. The probiotic strains have the natural proteolytic ability that is enough to justify their use for altering the milk proteins without being harmful. By doing so, the direct use of mammalian milk-based and plant-based infant formulas can prove way too advantageous for the little humans (Bu et al., 2013).

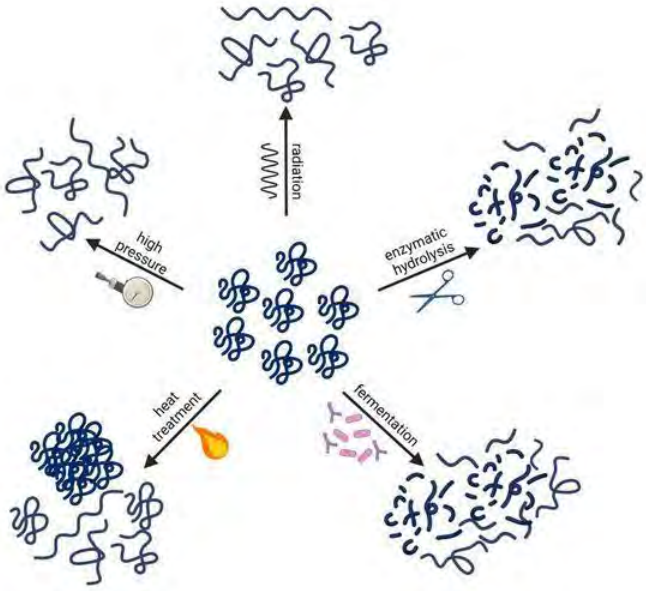


Figure 3 Common protein structural modifications induced by different processing technologies (Maryniak et al., 2022)

CHAPTER 03

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1. Strain Revival

The probiotics used in the research process include three bacterial strains i.e. *Streptococcus thermophilus* QAUSTN63, *Lactobacillus delbrueckii* subsp. *bulgaricus* QAULDN14, *Lactococcus lactis* QAULLNA8, and *Geotrichum candidum* QAUGC01 (PRJNA523005).

3.1.1. Media Preparation

The selective media for the growth of *Streptococcus thermophilus* QAUSTN63 and *Lactobacillus delbrueckii* subsp. *bulgaricus* QAULDN14 is MRS and that for *Lactococcus lactis* QAULLNA8 is Tryptic Soy Broth (TSB). In addition, the fungal strain *Geotrichum candidum* QAUGC01 (PRJNA523005) uses Oxytetracycline Glucose Agar (OGA). The media calculation for 1000 ml dH_2O of MRS and OGA is as follows:

3.1.2. Enrichment in Broth

10 ml broth was prepared for each bacterial and fungal strain. The broth along with 4 wrapped test tubes were then subjected to sterilization in an autoclave at 121°C for 15 min. After sterilization, the broth was allowed to cool, and the test tubes were allowed to dry in a laminar flow hood. The broth was poured into each test tube and inoculated with thawed strains that were preserved in glycerol and stored at -80°C containing bacterial strains were placed in an anaerobic chamber in a shaker incubator at 37°C for 48-72 hrs. Likewise, *G. candidum* was incubated at 25°C in a shaker incubator for 48 hrs.

3.1.3. Culturing on Agar Plates

50 ml of each growth medium i.e. MRSA, TSA, and OGA were prepared and sterilized along with 8 petri plates. After sterilization, the plates were allowed to dry in a laminar flow hood and the media was poured into each plate. After solidification, 100µl of enriched cultures of *Streptococcus thermophilus* QAUSTN63 and *Lactobacillus delbrueckii* subsp. *bulgaricus* QAULDN14, *Lactococcus lactis* QAULLNA8, and *Geotrichum candidum* QAUGC01 (PRJNA523005) were poured on MRSA, TSA, and OGA respectively. The bacterial plates were incubated at 37°C for 48-72 hrs while fungal plates were incubated at 25°C for 48 hrs.

3.2. Strain Preservation

After obtaining the colonies on agar plates, strain was preserved in Eppendorf tubes at -80ºC. Eppendorf tubes and 40% glycerol were sterilized at 121ºC for 15min. After sterilization, each Eppendorf tube was filled with 800µl broth enriched with individual strains followed by addition of 200µl 40% glycerol in each tube. The tubes were sealed by using parafilm and were stored in Ultra low temperature at -80ºC.

3.3. Morphological Characterization

3.3.1. Colony Morphology

A hand lens was used to look at the morphological characteristics of the obtained colonies on the agar plate to initiate a step towards the identification and characterization of the isolated strain.

3.3.2. Gram Staining

Gram staining of the prepared culture was carried out to categorize the cells as Gram-positive or Gram-negative.

The bacterial cells primarily stained by Crystal violet (primary stain) may be decolorized by alcohol (decolorizer) and take the color of safranin (secondary stain), hence the Gram-negative bacteria. Other bacteria that are not decolorized and retain primary stain are referred to as Gram-positive bacteria.

Staining was carried out for both broth and colonies on agar plates. The incubated broth (one of each sample) was shaken gently, and a drop was put on the glass slide. A small amount of already grown cultures (one of each sample) was picked by a sterile inoculating loop and mixed with a drop of distilled water on another glass slide followed by the smear formation. The smear was allowed to dry and then the slides were flooded with primary dye i.e. crystal violet for 1 min after which it was gently washed off with tap water. Gram's iodine, i.e. chelating agent, was poured on the smear and was allowed to sit for 1 min followed by washing. Then, the decolorizer i.e. alcohol was poured for 30 sec followed by washing. Finally, counter stain viz. safranin was poured on each slide and washed off after 1 min. The slides were dried and subjected to microscopy.

3.3.3. Fungal Staining

For Fungal staining, a drop of Lactophenol Cotton Blue stain was dropped on the glass slide. A small portion of the fungal colony was placed gently on the dye with the help of a sterile inoculating loop. The smear was then covered with a glass coverslip and subjected to microscopy.

3.3.4. Microscopy

Microscopy was carried out using compound light microscope, with 100X oil lens (immersion oil), to view the cells at an increased magnification and resolving power.

The ordinary compound light microscope is called as bright field microscope because it forms an image against a bright background. The microscope consists of a sturdy metal body composed of a base and an arm to which the remaining parts are attached. A light source, either a mirror or an electric illuminator, is located at the base. Two focusing knobs, the fine and coarse adjustment knobs, are located on the arm and move either the stage or the nosepiece for focusing the image. The stage is positioned about halfway up the arm and holds microscope slides either by simple slide clip or mechanical stage clip. A mechanical stage allows the operator to move a slide around smoothly during viewing by use of stage control knobs. The curved upper part of the arm holds the body assembly, to which nosepiece, eyepieces or ocular lenses are attached. The image seen when viewing a specimen with a compound microscope is created by the objective and ocular lenses working together. Light from the illuminated specimen is focused by the objective lens, creating an enlarged image within the microscope.

3.4. Preparation of the Bacterial Consortium and *G. candidum* Suspension

As per the recommendation of FAO, 10^8 CFU of each probiotic bacterium and *G. candidum* were prepared using Mc Farland standard. After attaining the required turbidity, the bacteria were mixed to form bacterial consortium having 10^8 CFU of all three probiotics i.e. *Streptococcus thermophilus* QAUSTN63 and *Lactobacillus delbrueckii* subsp. *bulgaricus* QAULDN14, *Lactococcus lactis* QAULLNA8, whereas, the fungal suspension was kept as such.

3.4.1. Mc Farland Standard

Mc Farland standards are used as a reference to adjust the turbidity of bacterial and yeast suspensions so that a specific CFU count per mililiter of a sample is obtained. A McFarland Standard is a solution of barium chloride and sulfuric acid. The chemical reaction between these two results in the production of fine precipitates of barium sulfate. After shaking well, the turbidity of a McFarland Standard is visually comparable to a bacterial or yeast suspension of known concentration. McFarland turbidity standards are prepared by mixing various volumes of 1% sulfuric acid and 1% barium chloride to obtain solutions with specific optical densities (OD). By adjusting the volume of these two chemical reagents, McFarland standards of varying degrees of turbidity can be prepared which represent different bacterial and yeast density or cell count.

Table 1 Mc Farland's Standards for Bacterial and Yeast Suspensions

Mc Farland Standard (MFU)	1% $BaSO_4$ (ml)	1% H_2SO_4 (ml)	Approximate CFU Count (x 10^8 cells)
0.5	0.05	9.95	1.5×10^8
1	0.1	9.9	3.0×10^8
2	0.2	9.8	6.0×10^8
3	0.3	9.7	9.0×10^8
4	0.4	9.6	11.0×10^8
5	0.5	9.5	15.0×10^8

3.5. Sample Collection (Milk)

For the production of fermented milk products, milk samples were collected from three animal sources and two commercial sources. Milk of healthy cow, goat, and sheep (1 litre each) was collected from a dairy farm. Alongside this, skim (powdered) and soy milk (1 litre) were also purchased from a grocery store.

3.5.1. Fermentation of Milk Samples

As skim milk was in powdered form so four liquid arrangements of 250 ml each were prepared having 2%, 4%, 6%, and 8% skim milk. Plus, 250 ml of cow milk, goat milk, sheep milk, and soy milk were also taken and subjected to sterilization at 110°C for 30 min. After sterilization, milk

was placed in laminar flow hood and allowed to cool. Meanwhile, bacterial cultures and fungal cultures were prepared for inoculation.

3.5.2. Preparation of Bacterial Cultures

5% consortium i.e. 11.5 ml broth was taken in five arrangements and centrifuged at 8000 rpm for 5 min at ambient temperature. After centrifugation, the supernatant was discarded and the pellet was washed with sterile normal saline thrice. The supernatant was discarded again and pellet was kept for further use.

3.5.3. Preparation of Fungal Cultures

5% inoculum of GC01 i.e. 11.5 ml broth was taken in five arrangements and centrifuged at 10,000 rpm for 15 min at ambient temperature. After centrifugation, the supernatant was discarded and the pellet was washed with sterile normal saline thrice. The supernatant was discarded again and pellet was kept for further use.

Centrifugation of the bacterial and fungal broth was carried out to obtain pellets of the bacterial consortium and fungal cells.

The centrifuge works on the principle of centrifugal force that is generated by high-speed rotation. The high-speed rotation of the rotor accelerates the sedimentation speed of particles in the liquid and separates the particles on the basis of density difference between them.

In this way, the cells settled at the bottom (pellet) while the media was supposed to have collected in the liquid (supernatant).

3.5.4. Fermentation

The bacterial consortium was dispensed into the milk samples and the inoculated milk was incubated at 40^{o}C for 6-8 hrs until curd formation was observed.

After 8 hrs, the obtained curds were broken down using a sterile spatula until a homogenized solution was formed. Then, the pellets of *G. candidum* were inoculated in each milk sample and incubated at 25^{o}C for 48 hrs. After incubation, 0.5 ml of each fermented milk sample was subjected to FTIR Spectral Analysis.

3.6. Sample Preparation for Lyophilization

The fermented milk products were stirred well to obtain a homogenized mixture. After that, the samples were poured into sterile petri plates with each plate having 5 ml of milk. The plates were stored at -4°C for one day and shifted at -20°C afterward.

All the fermented milk samples were lyophilized to obtain the powdered product. Afterward, 0.1g of each sample was subjected to FTIR Spectral Analysis.

3.6.1. Lyophilizer/ Freeze-dryer

Lyophilizer or freeze-dryer is an equipment that carries out the cryodesiccation process. It is a dehydration process occurring at low temperatures and low pressure. After the sample gets frozen, ice crystals are removed by sublimation. The lyophilized product retains the majority of its original properties and extends product shelf-life. The freeze-dried products can be stored for 3-5 years at temperatures lower than -20°C.

3.6.2. Fourier-transform Infrared Spectroscopy (FTIR)

Fourier-transform Infrared Spectroscopy (FTIR) is a technique used to obtain an infrared spectrum of the transmission or absorption of certain of solids, liquids, and gases. An FTIR equipment has the ability to collect high-resolution spectral data over a wide spectral range. It can be a useful technique for quality control, detection of contamination, and identification of functional groups, etc.

3.7. Storage of the Lyophilized Milk Samples

The obtained powdered milk samples i.e. fermented powdered soy milk, fermented powdered skim milk, fermented powdered cow milk, fermented powdered sheep milk, and fermented powdered goat milk, were stored at three different temperatures i.e. 4°C, 25°C, and -18°C.

3.8. Microbiology of Lyophilized Milk Products

The microbiology of all five lyophilized fermented milk products viz. Soy milk, Skim milk, Cow milk, Sheep milk, and Cow milk, was carried out to check for the safety profile of each product. For this purpose, TSA, MRSA, PDA, OGA, EMB agar, and MacConkey agar were prepared and sterilized at 121°C for 15 min. After sterilization, each media was poured in sterile petri plates and allowed to solidify. Then, 1g of each lyophilized sample was diluted in 10 ml luke warm distilled

water and marked as stock solution. The stock solution was then serially diluted up to 10^{-6} dilutions. Afterward, 100µl of each dilution was spread on agar surface. The plates containing PDA and OGA were incubated at 25ºC for 48 hrs, TSA and MRSA in anaerobic chamber at 37ºC for 48 hrs, and EMB and MacConkey at 37 ºC for 24 hrs. After incubation, the plates were observed to look for any bacterial or fungal contamination.

3.9. Impact of Fermentation on the Nutritional Profiling of The Milk Samples

3.9.1. Proximate Analysis

Proximate analysis determines the quantity of individual constituents within a substance. Generally, proximate analysis calculates the content of moisture, ash, volatile matter, fats, proteins, etc.

AOAC (2000) guidelines were followed to carry out the proximate analysis of each milk sample before (100ml each) and after (100g each) lyophilization.

3.9.1.1. Determination of Moisture Content

5g of dried and 5 ml of liquid samples were put in china dishes. The dishes were then placed in a hot air oven set at 105±5ºC and samples were allowed to dry overnight. The process was continued until a constant final weight was achieved. Then, the percentage of moisture content was calculated using the following formula:

$$Moisture\ (\%) = \frac{Wt.\ of\ fresh\ sample - Wt.\ of\ dry\ sample}{Wt.\ of\ fresh\ sample} \times 100$$

3.9.1.2. Determination of Ash Content

5g of dried and 5 ml liquid samples were put in a pre-weighed charred crucible. The sample was incinerated at 550ºC in a furnace. Then, the percentage of ash content was calculated on the basis of the difference in the weight of the sample before and after incineration.

$$Ash\ (\%) = \frac{Weight\ of\ ash}{Weight\ of\ Sample} \times 100$$

3.9.1.3. Determination of Crude Protein

Kjeldahl's method was used to determine the protein content in each sample. 25ml of concentrated H_2SO_4 and a digestion mixture of K_2SO_4, $CuSO_4$, and $FeSO_4$ were used to digest 500 mg of dried and 500 µl liquid samples. 10 ml NaOH was used for the distillation of the digested material. Then, receiver having 4% solution of boric acid was used to collect the liberated NH_3. This step was followed by the titration of nitrogen content using H_2SO_4 till a golden-brown end point was achieved. The percentage protein content was determined was calculated by multiplication of nitrogen contents with 6.25 factor.

$$Crude\ Protein\ (\%) = Nitrogen\ (\%) \times 6.25$$

3.9.1.4. Determination of Total Fats

The fat content of milk samples was detected using Schmid-Bondsynski-Ratzlaff (SBR) method. 6g of dried and 6 ml liquid samples were taken and 20ml HCl was added to it. The samples were heated in a water bath. After some time, the solution formed was transferred to a separating funnel and mixed with 20 ml of ethyl alcohol. After the precipitation of protein, 50ml of both diethyl ether and petroleum ether were added and mixed for about a minute. Then, the ethereal layer was separated from the inorganic layer and was drawn off to a pre-weighed china dish. The ethereal layer was placed in hot air oven at 102°C for 30 minutes. After the complete evaporation of solvent, the final weight of the fats was recorded. Percentage fats was calculated using the following formula:

$$Fat\ (\%) = \frac{W_1 - W_2}{W_3} \times 100$$

3.9.1.5. Determination of Total Solids

To determine the total solids, 3g of dried and 3 ml liquid samples were put in pre-weighed china dish. The weight of chine dishes was determined again after placement of samples. Then, the china dishes were placed in hot air oven at 102°C for 45 minutes. Afterward, the china dishes were weighed again and following formula was used to calculate total solids in each milk sample:

$$Total\ Solids\ (\%) = \frac{Dry\ weight\ of\ ash}{Weight\ of\ fresh\ sample} \times 100$$

3.9.1.6. Determination of pH

(Liquid Milk Sample)

The pH of liquid milk was determined before and after the inoculation of GC01. This is because GC01 is a yeast-like fungus and can grow on acidic medium, increasing its pH in return. Also, one of the reasons for choosing *G. candidum* was its pH neutralizing capability. After the inoculation of bacterial consortium, the milk samples were allowed to ferment for 8 hrs, and the pH of each fermented milk product was determined using a pH meter.

Similarly, after the inoculation of *G. candidum* and incubation at its optimum conditions, the pH of milk samples was determined again.

(Powdered Milk Samples)

After lyophilization, pH of each milk sample was determined again by dissolving 1g sample in 5 ml sterile distilled water.

3.10. Viability of the Strains

3.10.1. Viability Check

250 ml of MRSA, TSA, and OGA was prepared and sterilized at 121ºC for 15min. After sterilization, the plates were allowed to dry followed by pouring and solidification of the media. Then, 1g of each lyophilized sample was diluted in 10 ml luke warm distilled water. The sample was allowed to sit for 15 min. Afterwards, 100µl of each sample was poured and spread on both bacterial (MRSA and TSA) and fungal (OGA) media. The bacterial plates were incubated in anaerobic chamber at 37ºC for 48-72 hrs. And, the fungal plates were incubated at 25ºC for 48 hrs.

3.10.2. The CFU Count

1g of each lyophilized sample was dissolved in sterile distilled water and was marked as stock solution. The stock solution was serially diluted upto 10^{-6} dilutions. Each dilution was plated on MRSA and OGA media. The MRSA plates were incubated in anaerobic incubator at 37ºC for 48-72 hrs. And, the OGA plates were incubated at 25ºC for 48 hrs. After incubation, the colonies on each plate were counted using a colony counter and CFUs were calculated using the following formula:

$$CFU/ml = \frac{(No.\ of\ colonies \times dilution\ factor)}{Volume\ of\ culture\ plated\ (ml)}$$

3.11. Probiotic Attribute Stability

After lyophilization, the cultures of bacteria and fungus were obtained from all five lyophilized products. These cultures were checked for their attribute stability after regular intervals (up to 3 months) in an attempt to make sure that they retain their probiotic potential after lyophilization.

3.11.1. Lactose Fermentation

The acid production property of the bacterial consortium was checked by subjecting it to lactose fermentation test by introducing lactose in growth media.

250 ml of MRS was prepared and 0.4g (2%) lactose was added to both media and sterilized at 121ºC for 15 min. After sterilization, a few drops of methyl red indicator were added in the media and stirred well. The media was poured in sterile petri plate and allowed to solidify. Obtained cultures of *G. candidum* and bacterial consortium cultures from each lyophilized product were streaked on the OGA and MRS agar respectively. The bacterial plates were incubated in an anerobic chamber at 37ºC for 48-72 hrs whereas, the fungal plates were incubated at 25ºC for 48 hrs. After incubation, the plates were observed for appearance of pink colonies on agar plates.

3.11.2. Curdling of Milk

The fermentation of dairy products, especially milk was tested for both bacterial consortium and fungus. 100ml raw milk was boiled at 95ºC for 30 min. After boiling, the milk was allowed to cool down and was divided in 10 parts, 10 ml each in test tubes. Then, 5 test tubes were inoculated with obtained cultures of *G. candidum* and the other five with bacterial consortium. The bacterial test tubes were incubated in anaerobic chamber at 42ºC and the fungal test tubes at 25ºC for 6-8 hrs. After incubation, milk was observed for presence of curds in it.

3.11.3. Growth at Different Temperatures

To determine the temperature tolerance of the bacterial consortium and fungal strain, they were grown at four different temperatures viz. 15ºC, 25ºC, 37ºC and 45ºC.

250ml of MRS broth and OGB were prepared and sterilized at 121ºC for 15 min. After sterilization, the broth was allowed to cool and each one was divided in 4 sets of 6 equal parts. Four vials of each media were sealed without inoculation and were marked as controls. The other four vials were inoculated with obtained bacterial consortium and GC01. The four pairs were labelled as A, B, C and D showing temperatures 15ºC, 25ºC, 37ºC and 45ºC respectively. Both the bacterial and fungal vials were capped and placed in four different shaking incubators (anaerobic for bacteria

only) with temperatures 15ºC, 25ºC, 37ºC and 45ºC respectively. After incubation, the cultures were subjected to spectrophotometric analysis after 24hrs, 48hrs and 72hrs in a sequence using a 96-well microtiter plate.

3.11.4. Growth at Different pH

To determine the pH tolerance of the bacterial consortium and fungal strain, they were grown at four different pH viz. 2, 4, 6, and 8.

250ml of MRS broth and OGB were prepared and sterilized at 121ºC for 15 min. After sterilization, the broth was allowed to cool and each one was divided in 4 sets of 6 equal parts. Four vials of each media were sealed without inoculation and were marked as controls. The other four vials were inoculated with obtained bacterial consortium and *G. candidum*. The four pairs were labelled as A, B, C and D showing pH 2,4,6, and 8 respectively. Both the bacterial and fungal vials were capped and placed in four different shaking incubators (anaerobic for bacteria only) with temperatures 37ºC and 25ºC respectively. After incubation, the cultures were subjected to spectrophotometric analysis after 24hrs, 48hrs and 72hrs in a sequence using a 96-well microtiter plate.

3.11.5. Growth at Different Salt (NaCl) Concentrations

To determine the NaCl tolerance of the bacterial consortium and fungal strain, they were grown at four different concentrations of NaCl viz. 2%, 4%, 6%, and 8%.

250ml of MRS broth and OGB were prepared and sterilized at 121ºC for 15 min. After sterilization, the broth was allowed to cool and each one was divided in 4 sets of 6 equal parts. Four vials of each media were sealed without inoculation and were marked as controls. The other four vials were inoculated with obtained bacterial consortium and *G. candidum*. The four pairs were labelled as A, B, C and D showing NaCl concentrations i.e. 2% (0.2g), 4% (0.4g), 6% (0.6g), and 8% (0.8g) respectively. Both the bacterial and fungal vials were capped and placed in four different shaking incubators (anaerobic for bacteria only) with temperatures 37ºC and 25ºC respectively. After incubation, the cultures were subjected to spectrophotometric analysis after 24hrs, 48hrs and 72hrs in a sequence using a 96-well microtiter plate.

3.11.6. Growth at Different Bile Salts Concentrations

To determine the bile salts tolerance of the bacterial consortium and fungal strain, they were grown at four different bile salt concentrations viz. 0.15%, 0.25%, 0.35%, and 0.45%.

250ml of MRS broth and OGB were prepared and sterilized at 121°C for 15 min. After sterilization, the broth was allowed to cool and each one was divided in 4 sets of 6 equal parts. Four vials of each media were sealed without inoculation and were marked as controls. The other four vials were inoculated with obtained bacterial consortium and GC01. The four pairs were labelled as A, B, C and D showing NaCl concentrations i.e. 0.15% (15mg), 0.25% (25mg), 0.35% (35mg), and 0.45% (45 mg) respectively. Both the bacterial and fungal vials were capped and placed in four different shaking incubators (anaerobic for bacteria only) with temperatures 37°C and 25°C respectively. After incubation, the cultures were subjected to spectrophotometric analysis after 24hrs, 48hrs and 72hrs in a sequence using a 96-well microtiter plate.

3.11.7. Spectrophotometry

Spectrophotometry of the samples at each time interval, as mentioned above, was carried out for determination of temp, pH, salt and bile salt tolerance, using a spectrophotometer.

This instrument works on the principle that each component absorbs or transmits light over a certain wavelength, so it measures the amount of light transmitted (T) or absorbed (A). A beam of monochromatic light is transmitted through a liquid culture. The cells suspended in the culture interrupt the passage of light, and the amount of light energy transmitted through the suspension is measured on the photoelectric cell and converted to electrical energy. The density of a cell suspension is expressed as absorbance or optical density (OD) and is directly proportional to the concentration of cells.

To determine the OD of bacterial consortium and *G. candidum* at specific temperature, pH, NaCl, and bile salts concentration, microtiter plate was used. Each well for both bacterial and fungal microtiter plate was filled with 200µl (per sample) of liquid cultures. This was then followed by obtaining OD at 600nm for bacterial consortium and 492nm for *G. candidum*.

3.11.8. Statistical Analysis

The results of the OD obtained from spectrophotometry of growth of bacterial consortium and *G. candidum* at different temperatures, pH, NaCl concentrations, and bile salts concentrations, were analyzed by using Two-way RM ANOVA. Any values of each parameter having a p-value less than 0.05 were reported as statistically significant. The p-values less than 0.05 were flagged with one asterisk, less than 0.01 with two asterisks, less than 0.001 with three asterisks, and less than 0.0001 with four asterisks on each graph.

3.11.9. Hemolytic Activity

For determining the hemolytic activity of the bacterial consortium and fungal strain, blood sample was added in media to check for any hemolysis (if present).

50ml of MRSA and OGA was prepared and sterilized at 121°C for 15 min. After sterilization, 5ml blood was added in each media and stirred well. The media was poured in sterile petri plate and allowed to solidify. Obtained cultures of the bacterial consortium and fungal strain from all products were streaked on the petri plate (with five sections). The bacterial petri plate was then placed in an anaerobic chamber in incubator at 37°C for 48-72 hrs whereas, the fungal plates were incubated at 25°C for 48 hrs. After incubation, α and β- hemolysis was observed.

3.11.9. Bile Salt Hydrolase (BSH) Activity

BSH activity was determined for the bacterial consortium and fungal strain to check for the BSH enzyme functioning that lowers the serum cholesterol levels in body.

100ml MRSA and OGA was prepared and 0.037% $CaCl_2$ was added to each media followed by sterilization at 121°C for 15 min. Meanwhile, the 0.3% bile salts (mixture of Sodium cholate and Sodium deoxycholate) were exposed to UV for 5 min. After sterilization, UV irradiated bile salt was added in each media and stirred well. The media was poured in sterile petri plates and allowed to solidify. Obtained cultures of the bacterial consortium and fungal strain from all products were streaked on the petri plates. The bacterial petri plates were then placed in an anaerobic chamber in incubator at 37°C for 48-72 hrs whereas, the fungal plates were incubated at 25°C for 48 hrs. Crystal formation was observed in case of the presence of BSH enzyme.

3.11.10. Proteolytic Activity

Proteolytic activity of the bacterial consortium and fungal strain was carried out to check for capability of protein breakdown in the media.

200ml distilled water was taken and divided into two parts i.e. 100ml each. 5% (10g) skimmed milk was added to one part and 2% (4g) agar was added to the other part. Both the parts were sterilized at 121°C for 15 min. After sterilization, both the parts were mixed well and poured in sterile petri plates i.e. five petri plates for bacterial consortium and five plates for *G. candidum*. After solidification, the obtained bacterial consortium and *G. candidum* cultures were spotted at different locations on the petri plates. The bacterial petri plates were then placed in an anaerobic chamber in incubator at 37°C for 48-72 hrs whereas, the fungal plates were incubated at 25°C for

48 hrs. Zone formation was observed after incubation to confirm the proteolytic activity of the strains.

3.11.11. Lipolytic Activity

(For Bacterial Consortium)

100ml of MRSA was prepared and 1% (1ml) Tween 80 was added to it followed by sterilization at 121ºC for 15 min. After sterilization, a few drops of methyl red indicator were poured in the media and stirred well. The media was poured into the petri plates followed by inoculation with bacterial consortium and incubation in an anaerobic chamber at 37ºC for 48-72 hrs. The plates were observed for color change after incubation.

3.11.12. Antimicrobial Activity

To check for the antimicrobial potential of the strain was checked against *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 14028, *E.coli* ATCC 25922, *Bacillus subtilis* ATCC 5230, *Staphylococcus aureus* ATCC 25923, *Clostridium sporogenes* ATCC 19404, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, and *Staphylococcus aureus* ATCC 6538

100ml nutrient agar was prepared and sterilized at 121ºC for 15 min. After sterilization prepared cultures of the pathogenic strains viz. *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 14028, *E.coli* ATCC 25922, *Bacillus subtilis* ATCC 5230, *Staphylococcus aureus* ATCC 25923, *Clostridium sporogenes* ATCC 19404, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, and *Staphylococcus aureus* ATCC 6538 were taken, and a small amount of colony was suspended in Eppendorf tubes containing sterile normal saline. The tubes were shaken gently and the cultures were streaked on agar plates using sterile cotton swabs. The plates and allowed to set for 15 min. Afterwards, 4 wells were made on each plate with sterile blue tip by pressing it against the media and the wells were sealed with agar. Metabolite Extraction: The freshly prepared bacterial consortium in MRSB and *G. candidum* in OGB were dispensed in sterile Eppendorf tubes and centrifuged at 10ºC for 15 min at 6000-7000 rpm.

The metabolite obtained in the supernatant was poured into the wells made in pathogenic plates by using yellow tips and allowed to set for 10-15 min. The petri plates were incubated at 37ºC for 24 hrs. Zone formation was observed after incubation to confirm antimicrobial activity of the

bacterial consortium and *G. candidum.*

3.11.13. Antibiotic Susceptibility (AST)

To look for the antibiotic susceptibility of the bacterial consortium, an antibiotic susceptibility test was carried out.

250 ml MH-agar was prepared and sterilized at 121ºC for 15 min. After sterilization, the media was poured into sterile petri plates and allowed to solidify. Prepared cultures of bacterial consortium were dispensed in normal saline and spread upon the agar plate. The antibiotic discs of Amoxycilin 10µg, Colistin Sulphate 10µg, Cefepime 30µg, Penicillin G 10µg, Chloramphenicol 30µg, Gentamycin 10µg, Tetracycline 30µg, Cephazolin 30µg, Cephalothin 30µg, and Amikacin 30µg were placed at equal spaces on the agar surface. The petri plates were placed in an anaerobic chamber at 37ºC for 48-72 hrs. Zone formation was observed after the completion of incubation.

3.11.14. Antifungal Susceptibility (AFST)

To look for the antifungal susceptibility of the *G. candidum*, an antifungal susceptibility test was carried out.

250 ml MH-agar was prepared and 2% glucose and 0.5µg/ml methylene blue dye (GMB) were added to it. The media was subjected to sterilization in an autoclave at 121ºC for 15 min. After sterilization, the media was poured into sterile petri plates and allowed to solidify. Prepared cultures of *G. candidum* were dispensed in normal saline and spread upon the agar plate. Then, sterile filter paper discs were placed at equal distances on the agar surface followed by spotting each disc with different antifungal agents i.e. Nystatin, Amphotericin B, Itraconazole, Voriconazole, and Fluconazole. The petri plates were incubated at 25ºC for 48 hrs. Zone formation was observed after the completion of incubation

CHAPTER 04

RESULTS

RESULTS

4.1. Strain Revival

4.1.1. In Broth

Strain revival of bacteria i.e. *Streptococcus thermophilus* QAUSTN63 and *Lactobacillus delbrueckii* subsp. *bulgaricus* QAULDN14, *Lactococcus lactis* QAULLNA8 was carried out by enrichment in MRSB separately. Whereas, the fungus *Geotrichum candidum* GC01(PRJNA523005) was grown in OGB. After incubation at the optimum conditions, all the bacteria showed growth by making the broth turbid. In addition, floating colonies of *G. candidum* were also observed on OGB's surface along with turbidity.

4.1.2. On Agar Plates

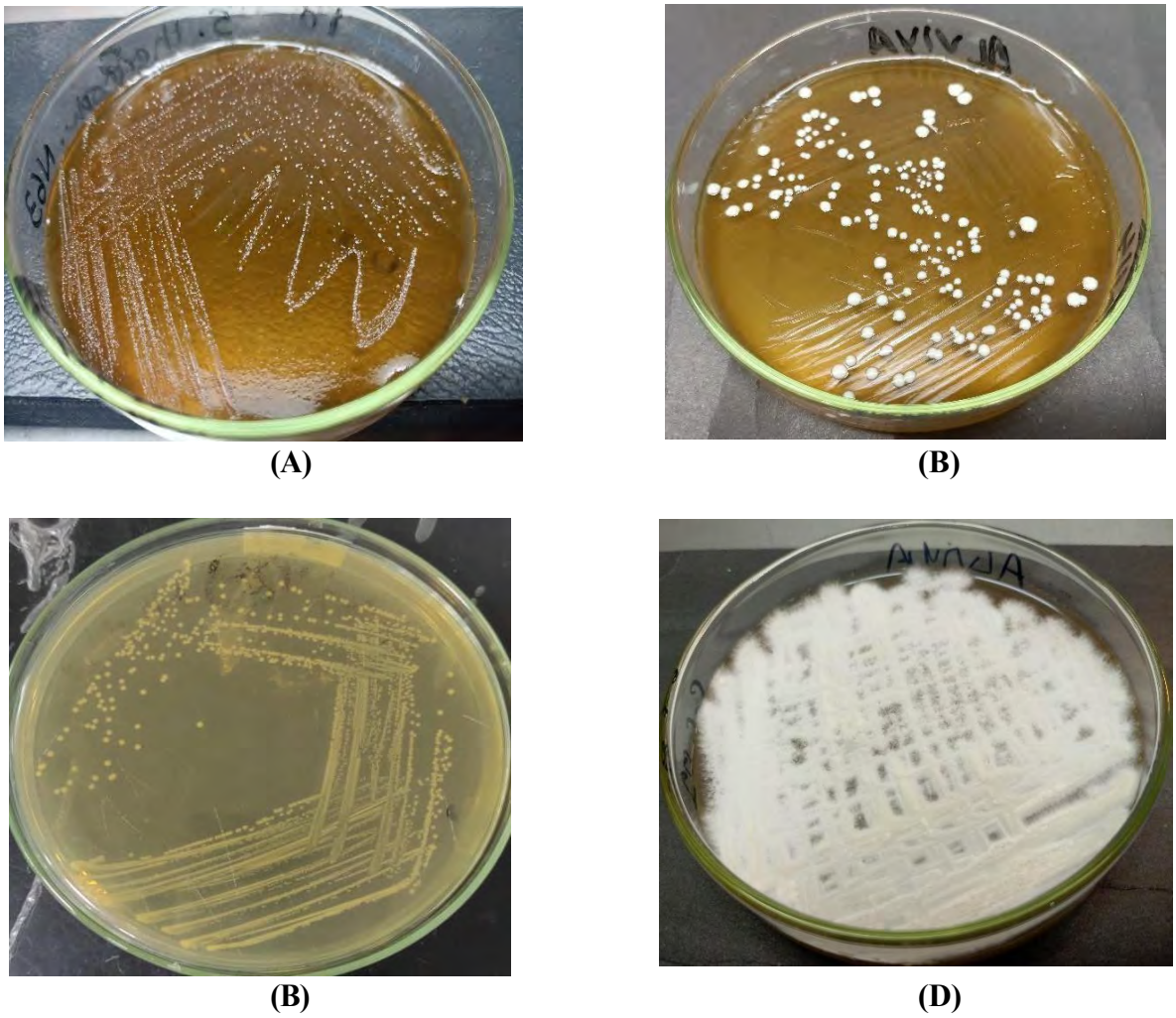
All the strains showed definite and distinct colonies on the agar plates. The colonies for *Streptococcus thermophilus* QAUSTN63 were small, white to creamy, round, non-mucoid. Whereas, *Lactobacillus delbrueckii* subsp. *bulgaricus* QAULDN14 showed medium, yellowish to white, non-mucoid colonies. In case of *Lactococcus lactis* QAULLNA8 showed small, white, mucoid colonies, present in the form of tiny clusters on the agar surface. Plus, the fungus *G. candidum* showed characteristic cotton-like or snowy white tocreamy, dry, suede-like colonies. Moreover, the colonies produced a nutty aroma that is also one of the identification properties of *G. candidum*.

4.2. Morphological Characteristics

4.2.1. Colony Morphology

Table 2 Colony morphology of the bacterial and fungal strains revived

Strain	Color	Mucoid/Non-mucoid	Shape
Lactobacillus delbrueckii QAULDN14	Yellowish White	Non-mucoid	Round
Lactococcus lactis QAULLNA8	White	Mucoid	Round
Streptococcus thermophilus QAUSTN63	Creamy white	Non-mucoid	Round
Geotrichum candidum QAUGC01	White/creamy	-	Flat



(A) **(B)**

(B) **(D)**

Figure 4(A) *Streptococcus thermophilus* QAUSTN63, (B) *Lactobacillus delbrueckii* QAULDN14, (C) *Lactococcus lactis* QAULLNA8, (D) *Geotrichum candidum* QAUGC01

4.2.2. Staining & Light Microscopy

The results of microscopy after staining the strains are summarized as follows.

4.2.2.1. Gram Staining of Bacterial Strains

All the bacterial species were stained **blue** showing their **Gram-positive** nature.

4.2.2.2. Cotton Blue Staining of Fungus

The staining of *G. candidum* depicted its dimorphic nature under the microscope. The funguscontained both hyphae and yeast-like cells.

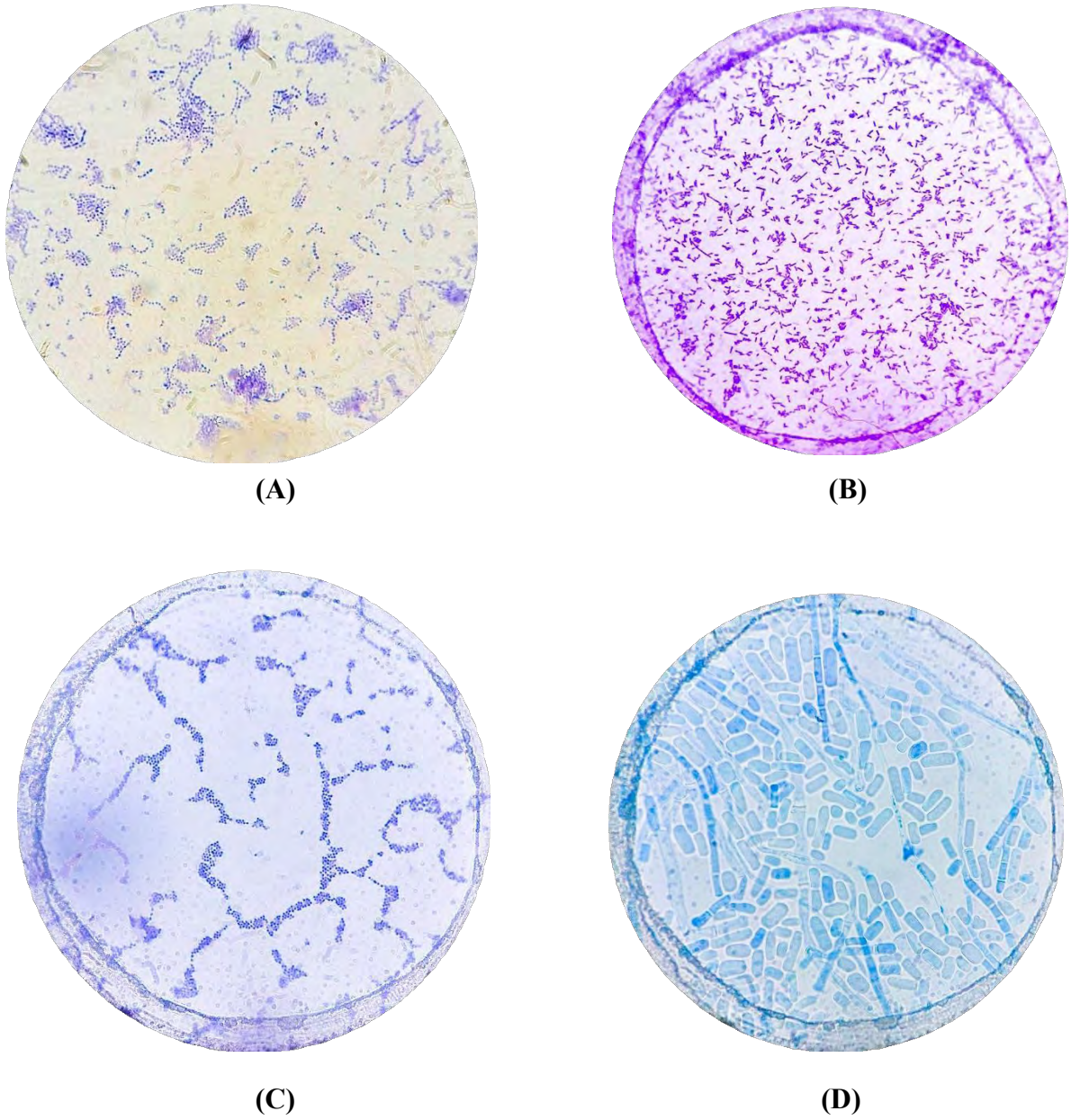


Figure 5 (A) Gram-positive cells of *Streptococcus thermophilus* QAUSTN63, (B*)* Gram-positive cells of *Lactobacillus delbrueckii* QAULDN14, (C) Gram-positive cells of *Lactococcus lactis* QAULLNA8, (D*)* *Dimorphic Geotrichum candidum* QAUGC01

Table 3 Microscopy results of the stained probiotic bacteria and fungus

Strain	Color	Cell Shape	Cell Arrangement
Streptococcus thermophilus QAUSTN63	Blue (Gram positive)	Round	Chains of cells
Lactobacillus delbrueckii QAULDN14	Blue (Gram positive)	Rod Shaped	Single rod-shaped cells
Lactococcus lactis QAULLNA8	Blue (Gram positive)	Round	Single/ clusters of cells
Geotrichum candidum QAUGC01	Blue	Cylindrical	Single cell/ hyphal arrangement (dimorphic)

4.3. Preparation of Bacterial Consortium and *G. candidum*

By using the Mc Farland standards, 10^8 CFUs of bacterial consortium (containing *Streptococcus thermophilus* QAUSTN63, *Lactobacillus delbrueckii* QAULDN14, and *Lactococcus lactis* QAULLNA8) and *G. candidum* were obtained for carrying out the fermentation ofmilk samples.

4.4. Fermentation of Milk Samples

(Bacterial Consortium)

The five selected milk samples viz. Soy milk, Skim milk, Cow milk, Sheep milk, and goat milkwere transformed into curd-like thick mass after 8 hrs of inoculation with bacterial consortium.

(G. candidum)

After inoculation of *G. candidum* and incubation for 48 hrs, a fermented solution of each milk samplewas obtained with visible growth of white colonies of *G. candidum*. The solution was again homogenized to distribute all the contents evenly throughout.

(A) (B)

Figure 6 (A) Fermentation of milk with bacterial consortium (B) Final fermented milk product after addition of *G. candidum*

4.5. The Lyophilized Fermented Milk Samples

The five fermented milk samples i.e. Soy milk, Skim milk, Cow milk, Sheep milk, and Goat Milk were transformed into powdered state after lyophilization. Each sample produced a finely dried yellowish white powder having characteristic fermented aroma with mild milky sweet flavor.

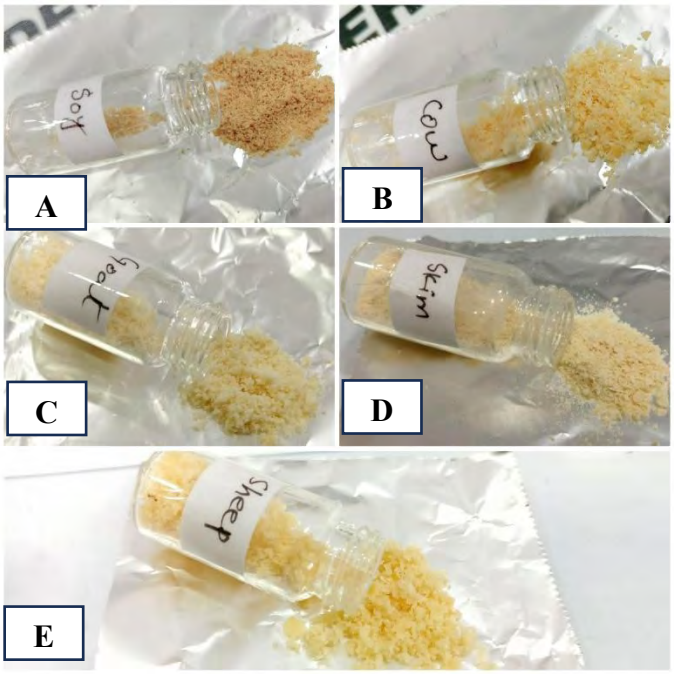


Figure 7 Fermented lyophilized products (A) Soy milk (B) Cow milk (C) Goat milk (D) Skim milk (E) Sheep milk

4.6. FTIR Spectral Analysis

4.6.1. Liquid Fermented Milk Samples

FTIR spectral analysis were carried out for final liquid fermented milk samples, i.e. before lyophilization, to check the contents of each sample.

The IR absorbance spectrum before lyophilization for Soy milk and goat milk, has showed strong appearance of alkenes. For Skim milk, the absorbance spectrum has given appearance of alkanes and conjugated alkenes. For Cow milk, the absorbance spectrum has given medium appearance of secondary amines and alkenes. For Sheep milk, alkenes, esters, and primary alcohols were observed.

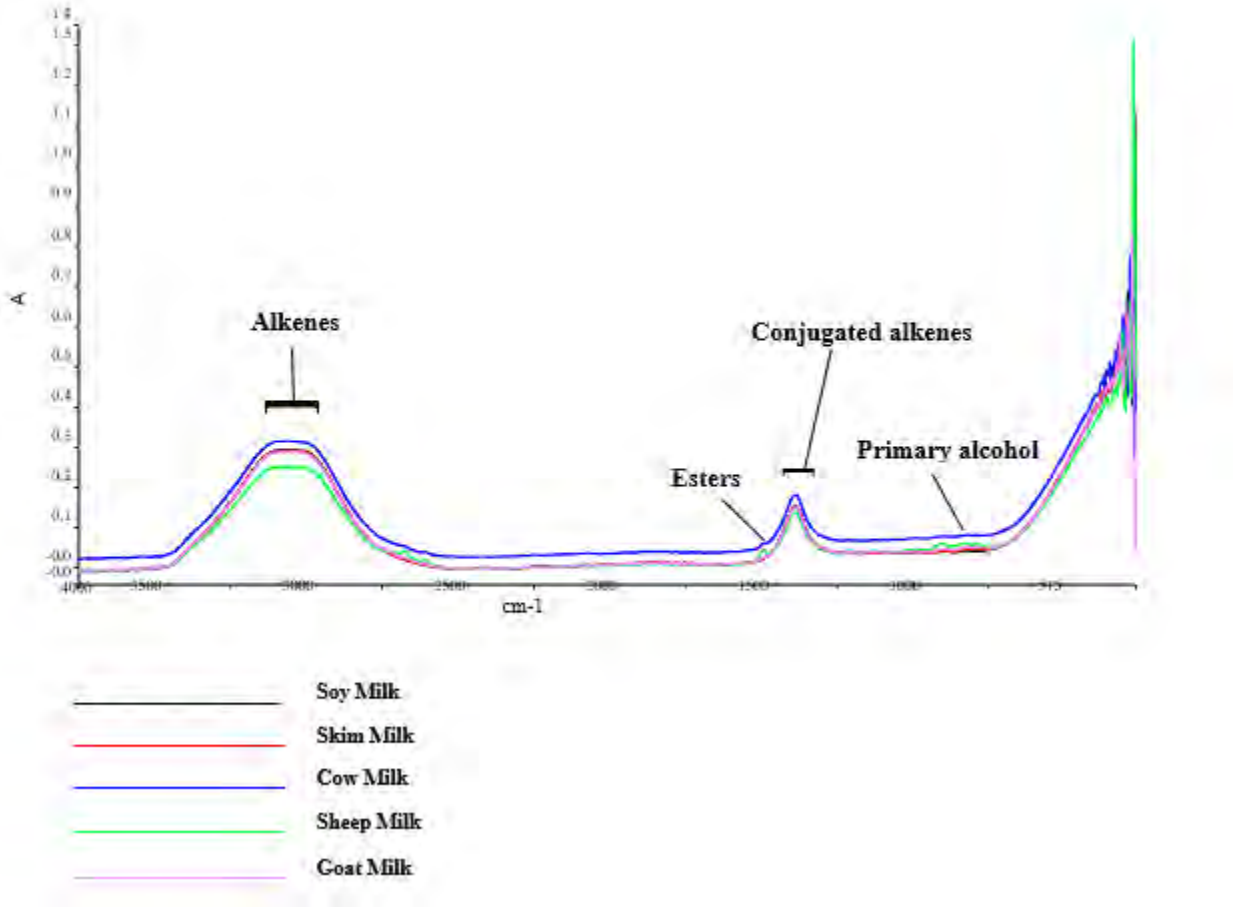


Figure 8 IR Absorbance spectrum of milk samples before lyophilization

4.6.2. Lyophilized Fermented Milk Products

FTIR spectral analysis were carried out for lyophilized fermented milk products to check whether or not the lyophilization process has altered the nutrient composition of any of theproducts. The IR absorbance spectrum after lyophilization for Soy milk showed strong appearance of carboxylic acids, amine salts, aliphatic ketones, nitro compounds, sulphonylchloride, alkyl aryl ethers, and anhydrides. For Skim milk, strong appearance of nitro compounds, sulphonyl chlorides, and alkyl aryl ethers. For Cow milk, strong appearance of alcohols, amine salts, aldehydes, sulfoxides, and nitro compounds were observed. For Sheep milk, strong appearance of alcohols, amine salts, α,β- unsaturated esters, nitro compounds, sulfates, and aldehydes were noted. For goat milk, strong presence of alcohols, nitro compounds, alkyl aryl ethers, and alkenes was observed.

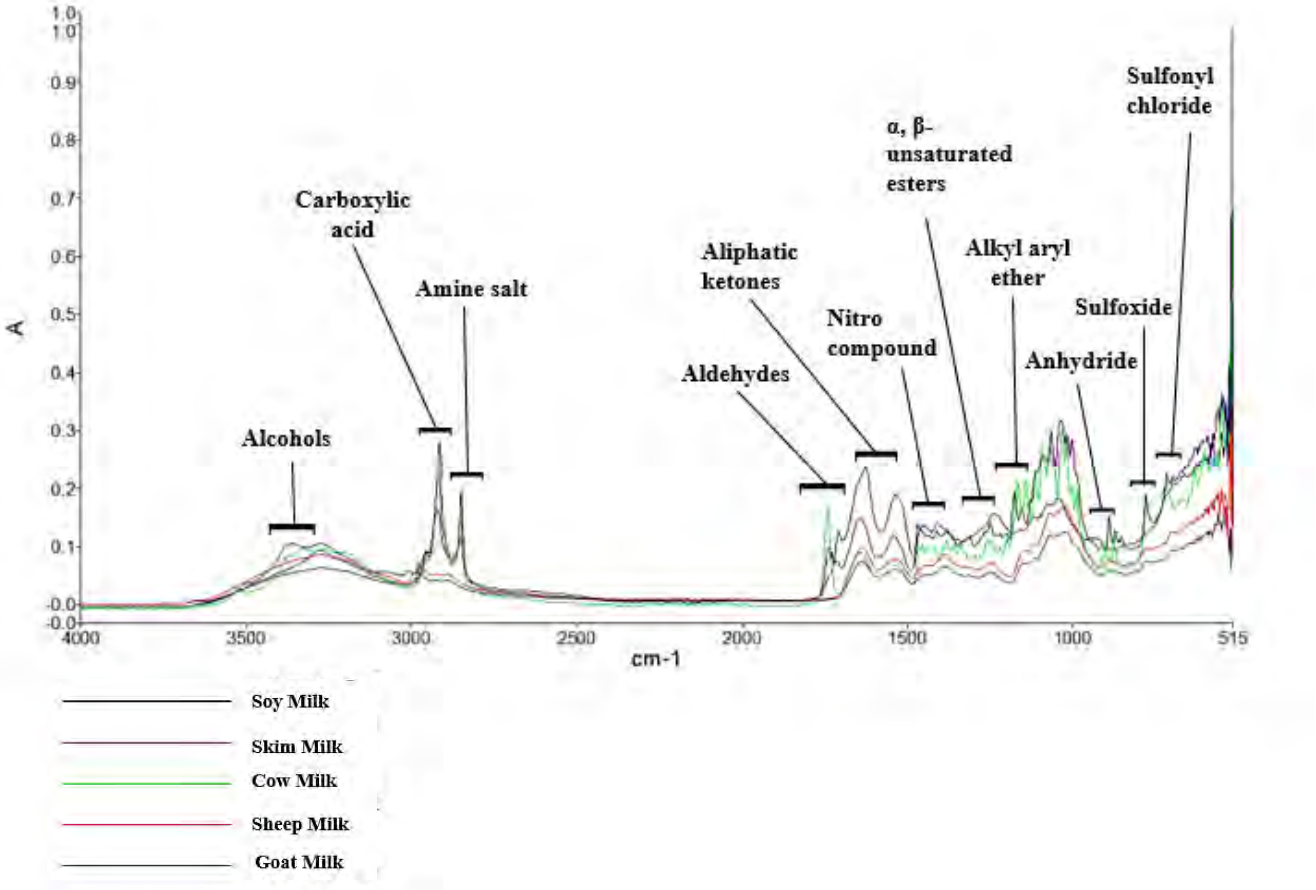


Figure 9 IR absorbance spectrum of milk samples after lyophilization

4.7. Microbiology of Lyophilized Milk Products

After incubation, the plates were observed for the presence of any bacterial and fungal

contamination. Fortunately, none of the plates of EMB and MacConkey agar showed growth of any gram-negative bacteria. In fact, the agar surface of each plate was clean with no growthat all. In addition, the plates of PDA and OGA showed growth of *G. candidum* only,with OGA surface having denser colonies. Plus, MRSA and TSA showed growth of three distinct whitish creamy colonies that were stained as gram-positive rods (*L.s delbrueckii*), clusters of cocci (*L. lactis*), and chains of coccoid cells (*S. thermophilus*). No other bacterial growth was observed proving that the products were not contaminated and contained inoculated bacterial and fungal species only.

4.8. Nutritional Profiling of The Milk Samples Before and After Lyophilization

The proximate analysis of each milk sample was carried out before and after lyophilization tocheck the intactness of nutrients in each product. The results were calculated using the mentioned formulas, and are summarized as follows:

4.8.1. Nutritional Profiling before Lyophilization

The results of proximate analysis before lyophilization and after fermentation are summarized as follows:

Table 4 Nutritional profiling (per 100 ml) of milk samples before lyophilization

Product/ Factor	Moisture	Ash	Crude protein	Total fats	Total solids	pH
Soy milk	82.91	0.99	5.0	0.7	9.4	6.7
Skim milk	94.72	0.388	1.72	Not detected	5.27	6.7
Cow milk	91.65	0.346	1.33	1.9	8.34	6.5
Sheep milk	80.55	1.053	3.72	8.5	19.44	6.5
Goat milk	87.41	1.030	3.55	3.7	12.58	6.55

4.8.2. Nutritional Profiling after Lyophilization

The results of proximate analysis after lyophilization are summarized as follows:

Table 5 Nutritional profiling (per 100g) of milk samples after lyophilization

Product/ Factor	Moisture	Ash	Crude protein	Total fats	Total solids	pH
Soy milk	0.45	5.4	27.01	26.19	96.55	6.6
Skim milk	0.18	5.31	24.58	Not detected	95.82	6.8
Cow milk	0.41	5.34	24.03	27.89	96.59	6.7
Sheep milk	0.54	5.48	25.22	29.07	96.82	6.6
Goat milk	0.81	5.37	25.02	26.76	96.63	6.6

4.9. pH

Table 6 pH of the fermented milk samples detected after the inoculation of bacterial consortium, *Geotrichum candidum*, and lyophilization respectively

Product	pH of Raw Milk	Fermentation with Bacterial Consortium	After Inoculation of *G. candidum*	After lyophilization
Soy Milk	6.7	5.85	6.7	6.6
Skim Milk	6.5	5.39	6.7	6.8
Cow Milk	6.6	5.6	6.5	6.7
Sheep Milk	6.6	5.20	6.5	6.6
Goat Milk	6.7	5.7	6.55	6.6

4.110. Viability of the Strains

4.10.1. Viability Check

The plates of MRSA and TSA showed growth of three distinct whitish creamy colonies that were stained as gram-positive rods (*Lactobacillus delbrueckii*), clusters of cocci (*Lactococcuslactis*), and chains of coccoid cells (*Streptococcus thermophilus*). This proved that the bacterialstrains were viable after lyophilization. Moreover, the plates of OGA showed appearance of characteristic *G. candidum* colonies proving its viability too.

4.10.2. The CFU Count

The serial dilution of each sample showed 10^8 CFUs of both bacterial consortium and *G. candidum* on average at the dilution 10^5. This proved that there was negligible to no change in the probiotic count in the products after lyophilization.

4.11. Probiotic Characterization

(Note: The attributes stability was checked for bacterial consortium and G. candidum isolated from each lyophilized milk product. Only one picture has been used as a reference image to show the results)

4.11.1. Lactose Fermentation

(Bacterial Consortium)

Formation of **pink colonies** was observed which is indicative of the production of acid after fermentation of lactose present in the media. The methyl red indicator changes its color from **orange** to **pink** in the presence of acid i.e. low pH. Hence, the Bacterial consortium gave a **positive** lactose fermentation test.

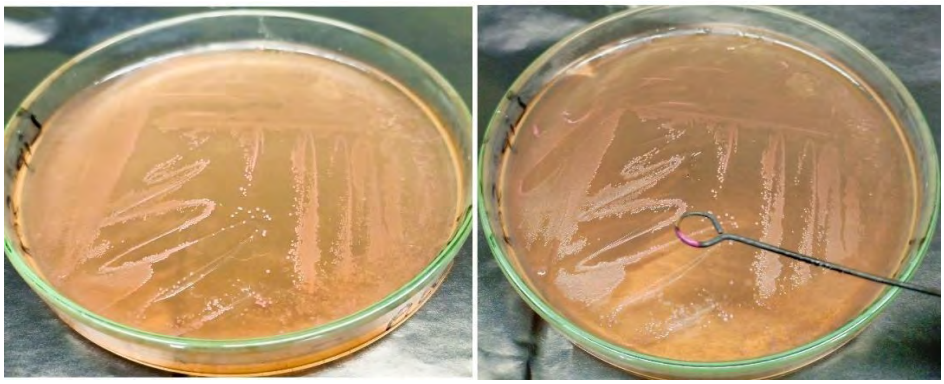


Figure 10 Appearance of pink colonies of bacterial consortium giving positive results for lactose fermentation test

(Geotrichum candidum)

The fungal strain *G. candidum* does not possess the ability to ferment lactose therefore it gave a **negative** lactose fermentation test.

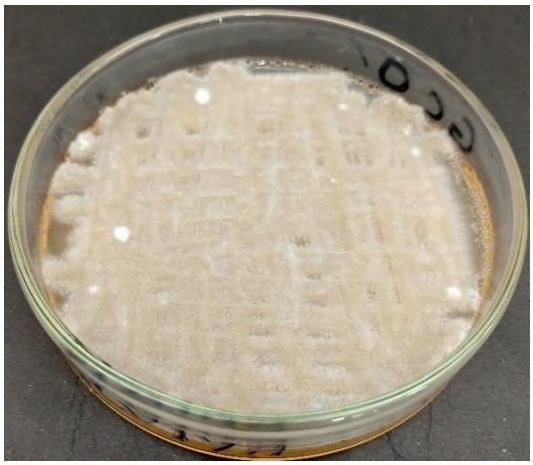


Figure 11 Negative lactose fermentation test for *G. candidum* (no appearance of pink colonies)

4.11.1. Curd Formation

The milk had been curdled by the bacterial consortium giving a **positive** milk fermentation andcurd formation test. However, *G. candidum* is a lactose non-fermenter and therefore it cannot ferment lactose. Hence, it did not form any curds in milk.

Figure 12 Curd Formation in milk by bacterial consortium

4.12. Growth at Different Temperatures

(Bacterial Consortium)

The growth of bacterial consortium, isolated from lyophilized soy milk, skim milk, cow milk,sheep milk, and goat milk, was observed at different temperatures viz. 15°C, 25°C, 37°C, and40°C showed an overall rising trend. However, the growth was observed best at 37°C, also declared as the significant value as per the results of statistical analysis (ANOVA).

Hence, 37°C is the optimum temperature for the growth of consortium isolated from every sample. The graph for every consortium's activity is as follows:

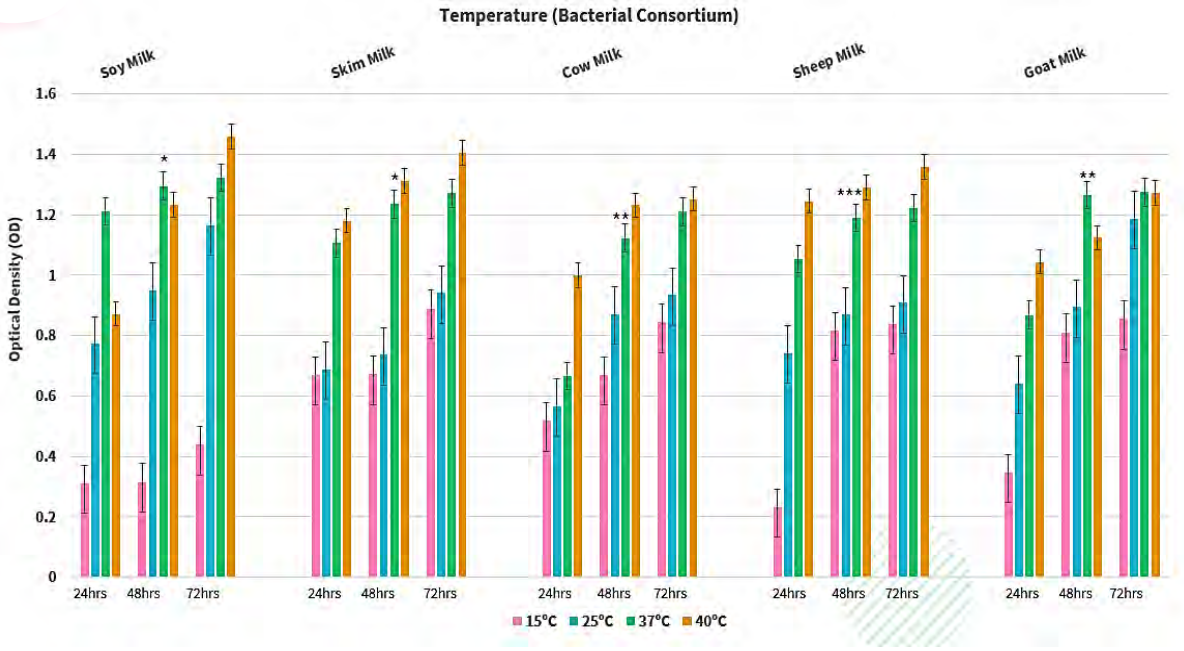


Figure 13 Growth of bacterial consortium at different temperatures

(Geotrichum candidum)

The growth of *G. candidum*, isolated from lyophilized soy milk, skim milk, cow milk, sheep milk, and goat milk, was observed at different temperatures viz. 15°C, 25°C, 37°C, and 40°C showedan overall rising trend. However, the growth was observed best at 25°C, also declared as the significant value as per the results of statistical analysis (ANOVA). Hence, 25°C optimum temperature for the growth of *G. candidum* isolated from every sample. The graph for every *G. candidum*'s activity is as follows:

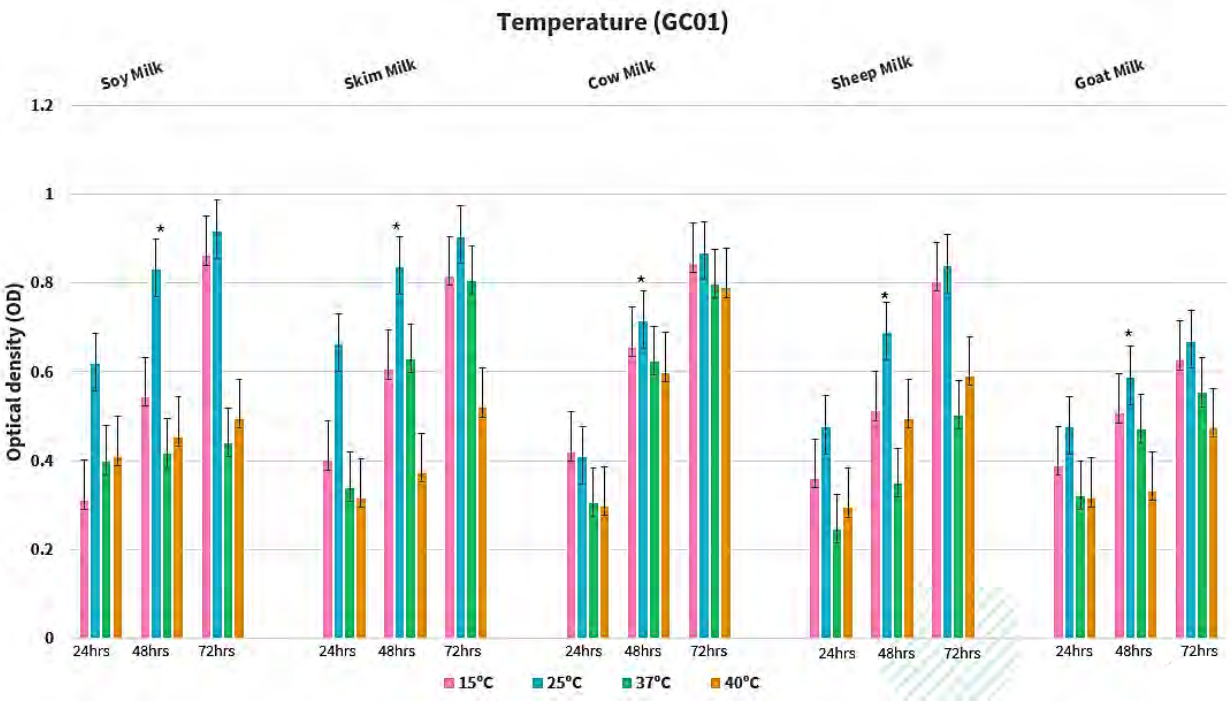


Figure 14 Growth of *Geotrichum candidum* at different temperatures

4.13. Growth at Different pH

(Bacterial Consortium)

The growth of bacterial consortium, isolated from lyophilized soy milk, skim milk, cow milk,sheep milk, and goat milk, was observed at different temperatures viz. 2,4,6, and 8 showed anoverall rising trend. However, the growth was observed best at 6, also declared as the significant value as per the results of statistical analysis (ANOVA). Hence, 6 is the optimum pH for the growth of consortium. The graph for every consortium's activity is follows:

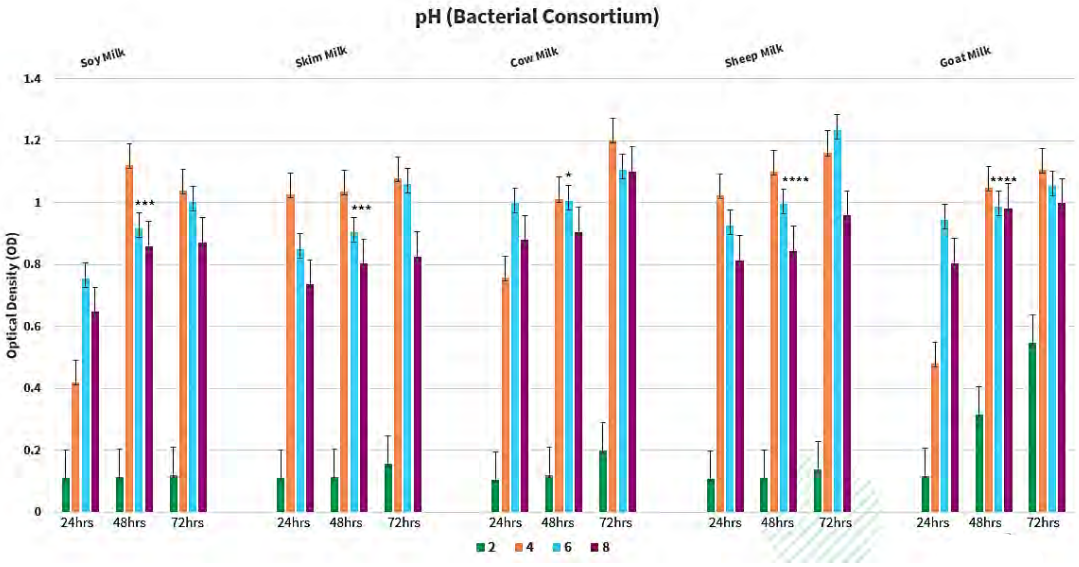


Figure 15 Growth of bacterial consortium at different pH

(Geotrichum candidum)

The growth of *G. candidum*, isolated from lyophilized soy milk, skim milk, cow milk, sheep milk, and goat milk, was observed at different pH viz. 2,4,6, and 8 showed an overall rising trend. However, the growth was observed best at 4, also declared as the significant value as per the results of statistical analysis (ANOVA). Hence, 4 is the optimum pH for the growth of *G. candidum*. The graph for every *G. candidum* 's activity is as follows:

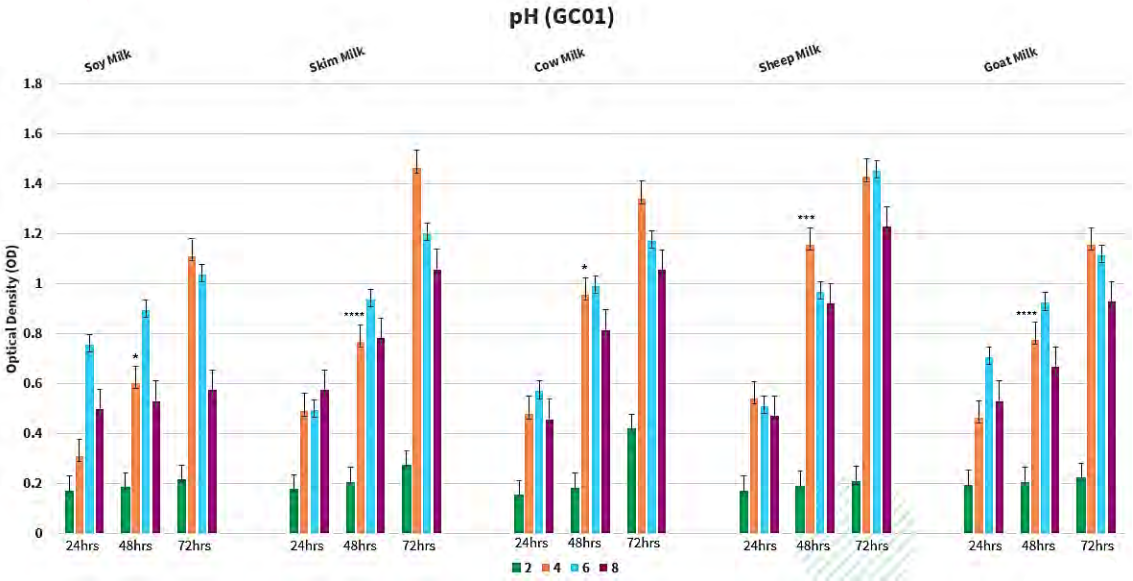


Figure 16 Growth of *Geotrichum candidum* at different pH

4.14. Growth at Different Salt (NaCl) Concentrations

(Bacterial Consortium)

The growth of bacterial consortium, isolated from lyophilized soy milk, skim milk, cow milk,sheep milk, and goat milk, was observed at different NaCl viz. 0.15%, 0.25%, 0.35%, and 0.45% showed an overall rising trend showing strain stability at all concentrations. However, best growth was observed at the concentration 8%, also declared as the significant value as per the results of statistical analysis (ANOVA). The graph of the activities presented by consortium from each sample is as follows:

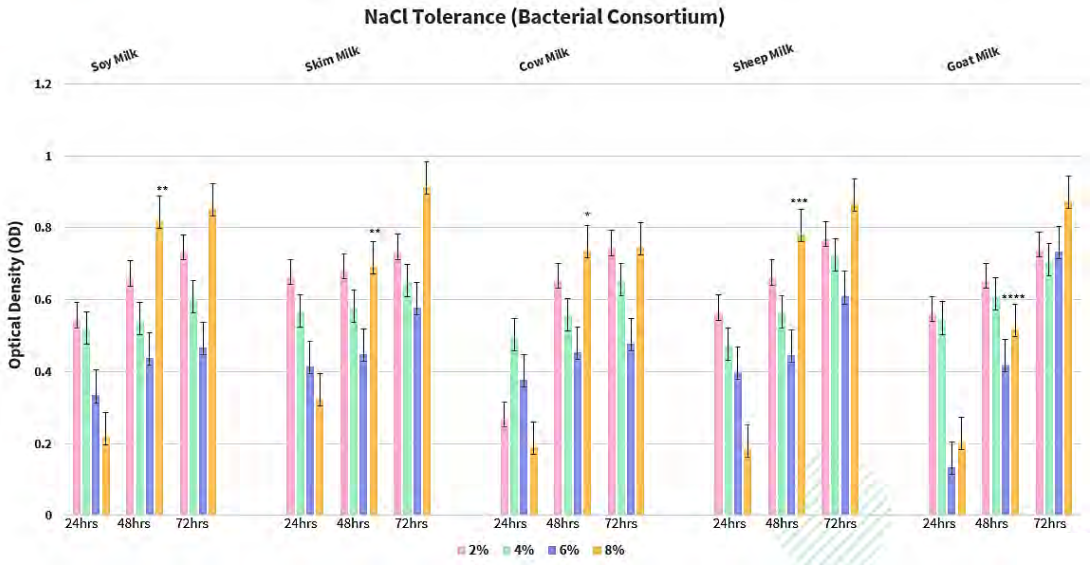


Figure 17 Growth of bacterial consortium at different NaCl concentrations

(Geotrichum candidum)

The growth of *G. candidum*, isolated from lyophilized soy milk, skim milk, cow milk, sheep milk, and goat milk, was observed at different NaCl viz. 2%, 4%, 6%, and 8% showed an overall rising trend showing strain stability at all concentrations. However, best growth was observedat the concentration 2% also declared as the significant value as per the results of statistical analysis (ANOVA).The graph of the activity shown by *G. candidum* from each sample is as follows:

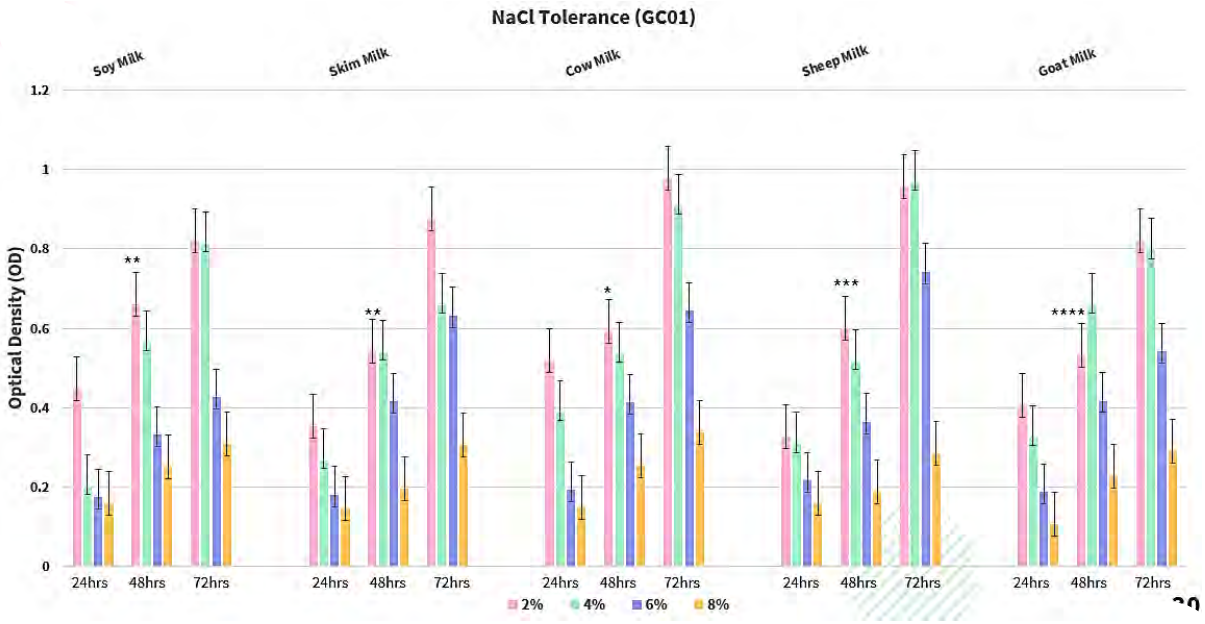


Figure 18 Growth of *Geotrichum candidum* at different NaCl concentrations

4.15. Growth at Different Bile Salt Concentrations

(Bacterial consortium)

The growth of bacterial consortium, isolated from lyophilized soy milk, skim milk, cow milk,sheep milk, and goat milk, was observed at different bile salts concentration viz. 0.15%, 0.25%,0.35%, and 0.45% showed an overall rising trend showing strain stability at all concentrations.However, best growth was observed at the concentration 0.35%, also declared as the significant value as per the results of statistical analysis (ANOVA).

The graphs of the activities presented by consortium from each sample are as follows:

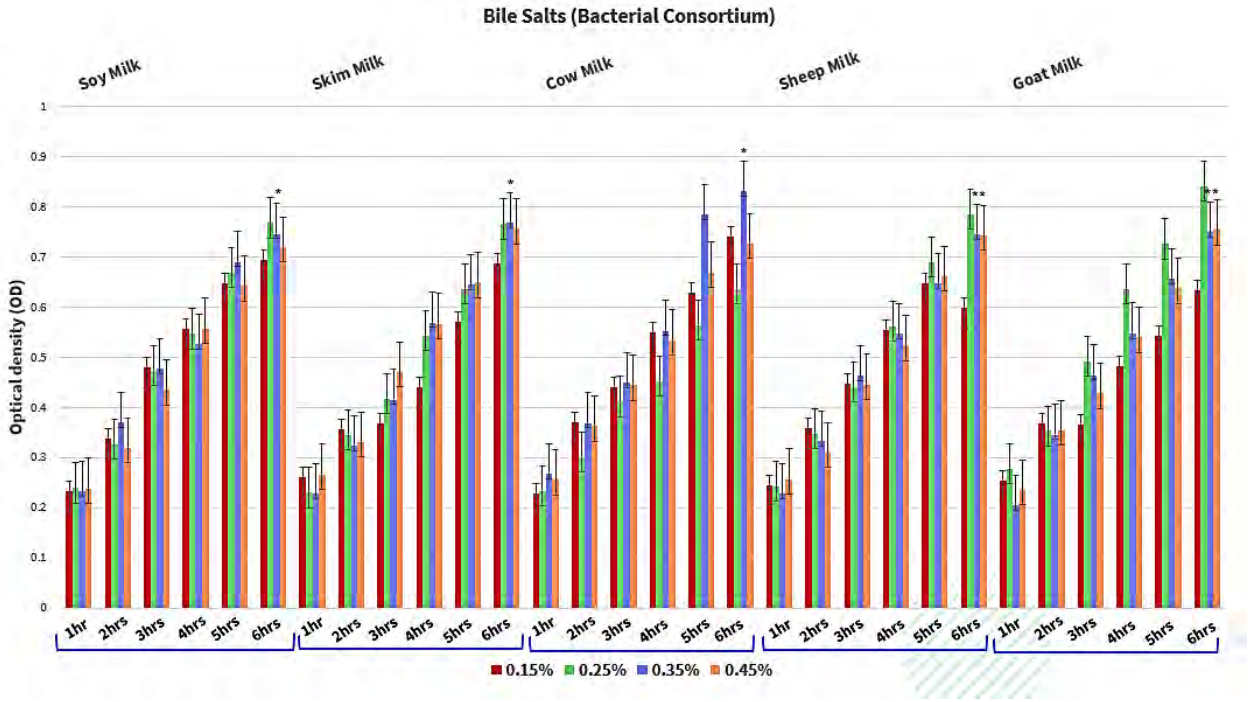


Figure 19 Growth of bacterial consortium at different bile salts concentration

(Geotrichum candidum)

The growth of *G. candidum* isolated from lyophilized soy milk, skim milk, cow milk, sheep milk, and goat milk, was observed at different bile salts concentration viz. 0.15%, 0.25%, 0.35%, and 0.45% showed an overall rising trend showing strain stability at all concentrations. However, best growth was observed at the concentration 0.25%, also declared as the significant value as per the results of statistical analysis (ANOVA). The graphs of the activities presented by consortium from each sample are as follows:

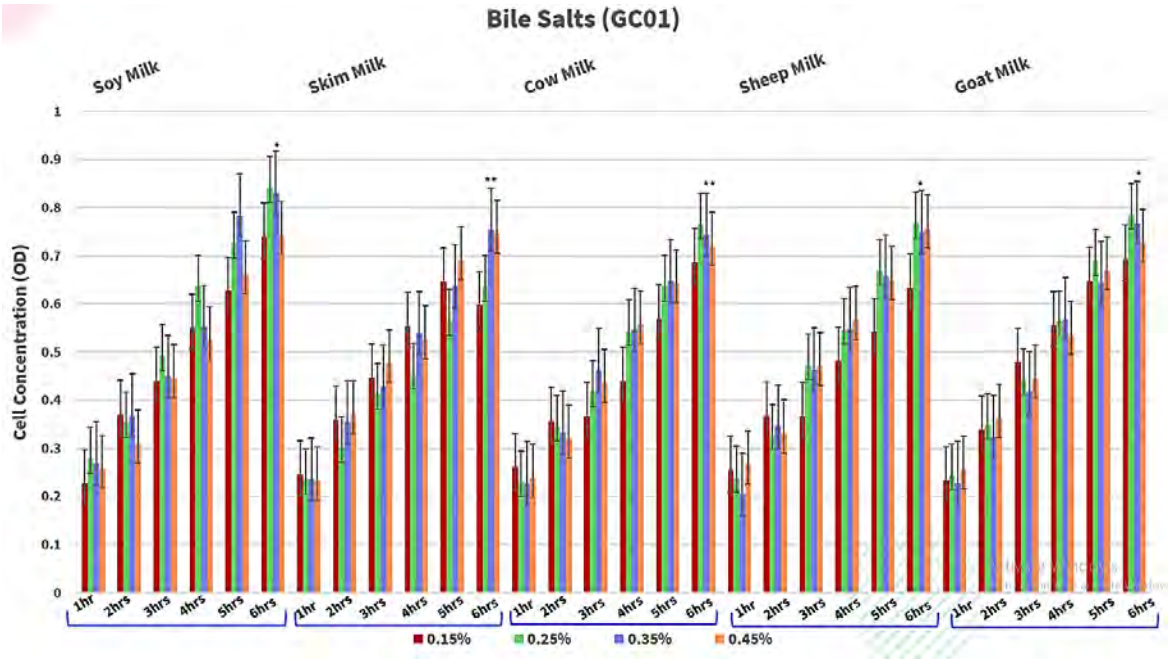


Figure 20 Growth of *G. candidum* at different bile salts concentration

4.16. Hemolytic Activity

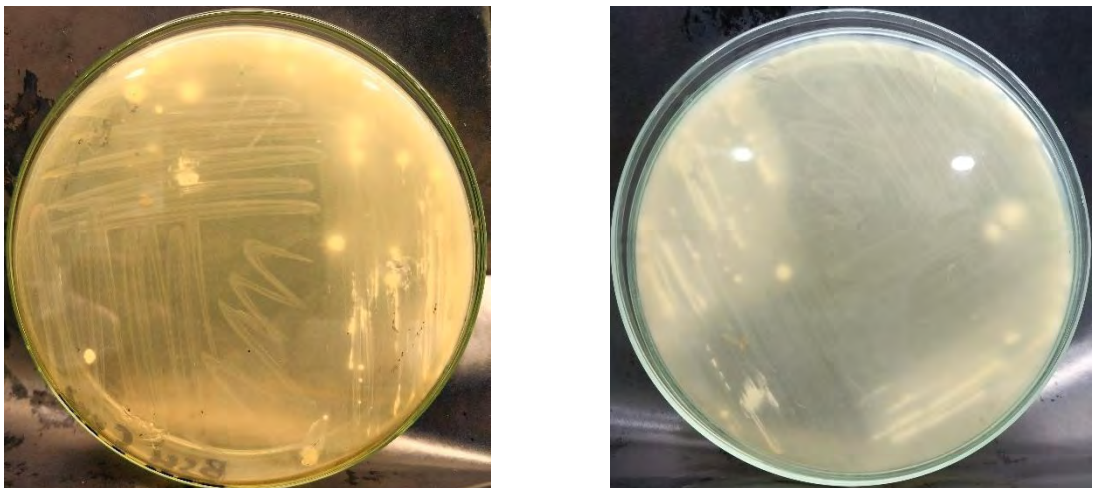
The bacterial consortium as well as *G. candidum* gave **gamma hemolysis** of blood. The colonies depicted discoloration i.e. brownish color showing no hemolytic capability.

(A) **(B)**

Figure 21 Gamma hemolysis shown by (A) Bacterial consortium (B) *G. candidum*

4.17. BSH Activity

The Bile Salt Hydrolase activity test for bacterial consortium showed crystal formation in the media giving a **positive** result for BSH activity.

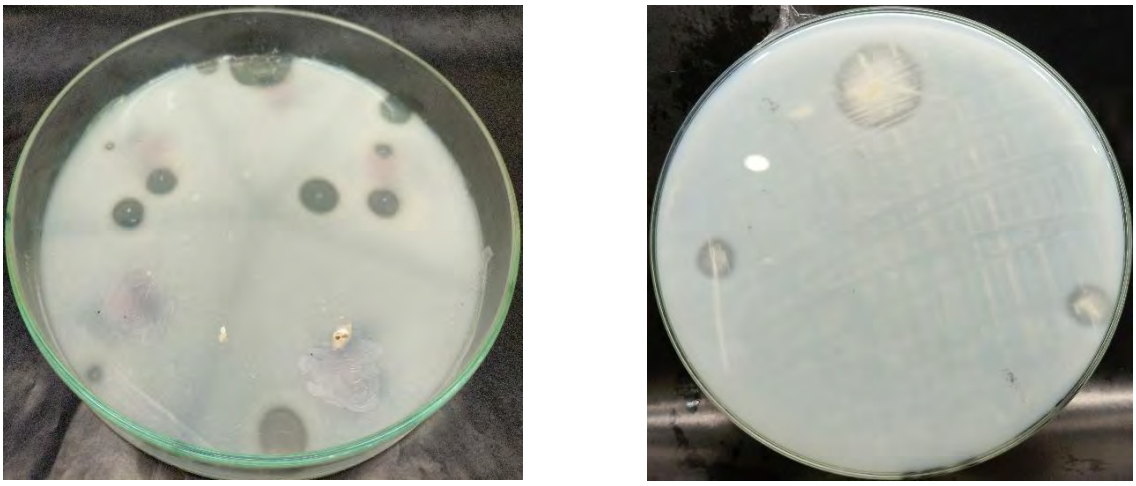


(A) **(B)**

Figure 22 Precipitation around the colonies of (A) Bacterial Consortium (B) *Geotrichum candidum* giving positive BSH activity

4.18. Proteolytic Activity

The proteolytic activity for both bacterial consortium and *G. candidum* showed **positive** result by formation of definite zones in skim milk agar media, i.e. all the strains possess proteolytic activity.



(A) **(B)**

Figure 23 Clear zone formation around the colonies of (A) Bacterial consortium and (B) *G. candidum showing* positive proteolytic activity

Table 7 Diameter of zone of inhibition as a result of proteolytic activity of bacterial consortium and *Geotrichum candidum*

Pathogen	Diameter of Zone of Inhibition	
	Bacterial Consortium	*G.* candidum
Soy Milk	0.3-0.4 cm	0.8 cm
Skim Milk	0.8-1 cm	1.2 cm
Cow Milk	0.5-0.8 cm	1.2 cm
Sheep Milk	0.9-1.7 cm	2.1 cm
Goat Milk	0.5-1.2 cm	2 cm

4.19. Lipolytic Activity

The extracellular lipases formed by the bacteria in consortium break down Tween 80 into freefatty acids causing a change in the pH of the media. The indicator, methyl red, therefore changes the color at low pH from **orange to pink.** Hence, pink colonies were formed by the bacterial consortium isolated from each milk sample showing a **positive** result for lipolytic activity.

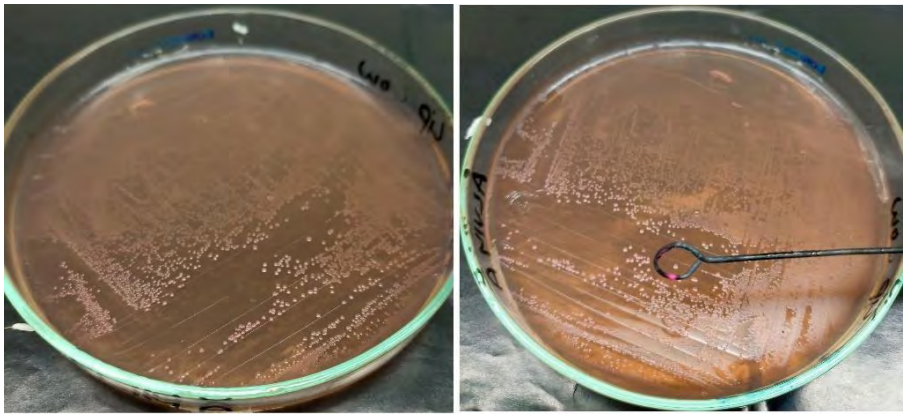


Figure 24 Appearance of pink colonies of bacterial consortium giving positive results for lipolytic activity

4.20. Antimicrobial Activity

(Bacterial Consortium)

The bacterial consortium showed antimicrobial activity against *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 14028, *E. coli* ATCC 25922, *Bacillus subtilis* ATCC5230, and *Staphylococcus aureus* ATCC 25923. However, it had no antimicrobial potential against

Clostridium sporogenes ATCC 19404, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, and *Staphylococcus aureus* ATCC 6538 depicted by no zone formation around the agar wells.

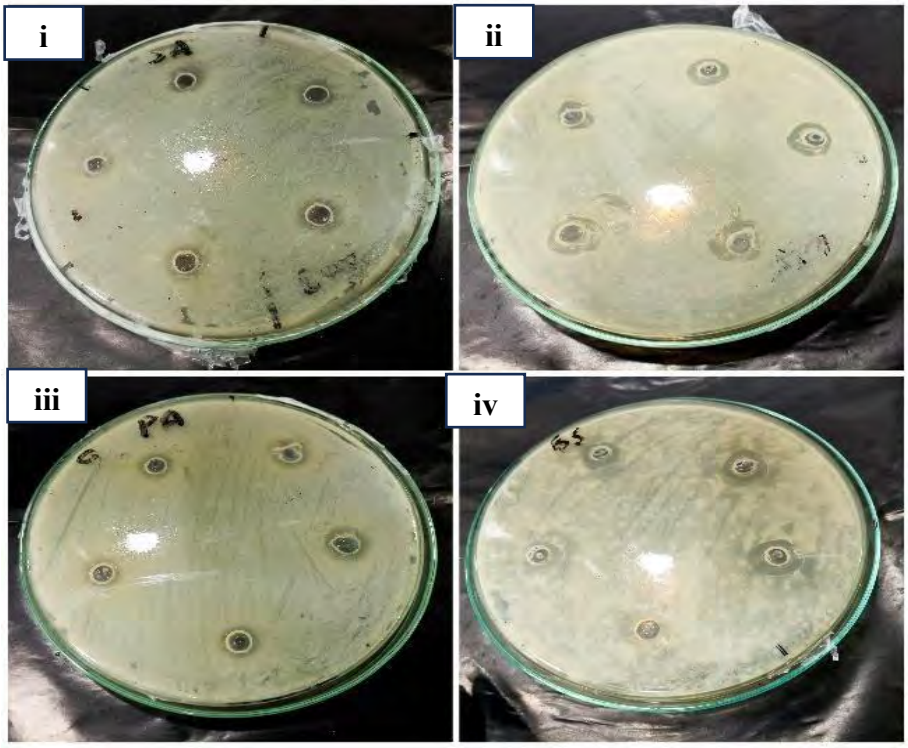


Figure 25 The antimicrobial metabolites of bacterial consortium forming zones of inhibition around the agar wells of (i) *Staphylococcus aureus* ATCC 25923 (ii) *E. coli* ATCC 25922 (iii) *Pseudomonas aeruginosa* ATCC 27853 (iv) *Bacillus subtilis* ATCC 523

(Geotrichum candidum)

On the other hand, *G. candidum* showed strong antimicrobial activity against *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 14028, *E. coli* ATCC 25922, *Bacillus subtilis* ATCC 5230, *Staphylococcus aureus* ATCC 25923, *Clostridium sporogenes* ATCC 19404, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, and *Staphylococcus aureus* ATCC 6538.

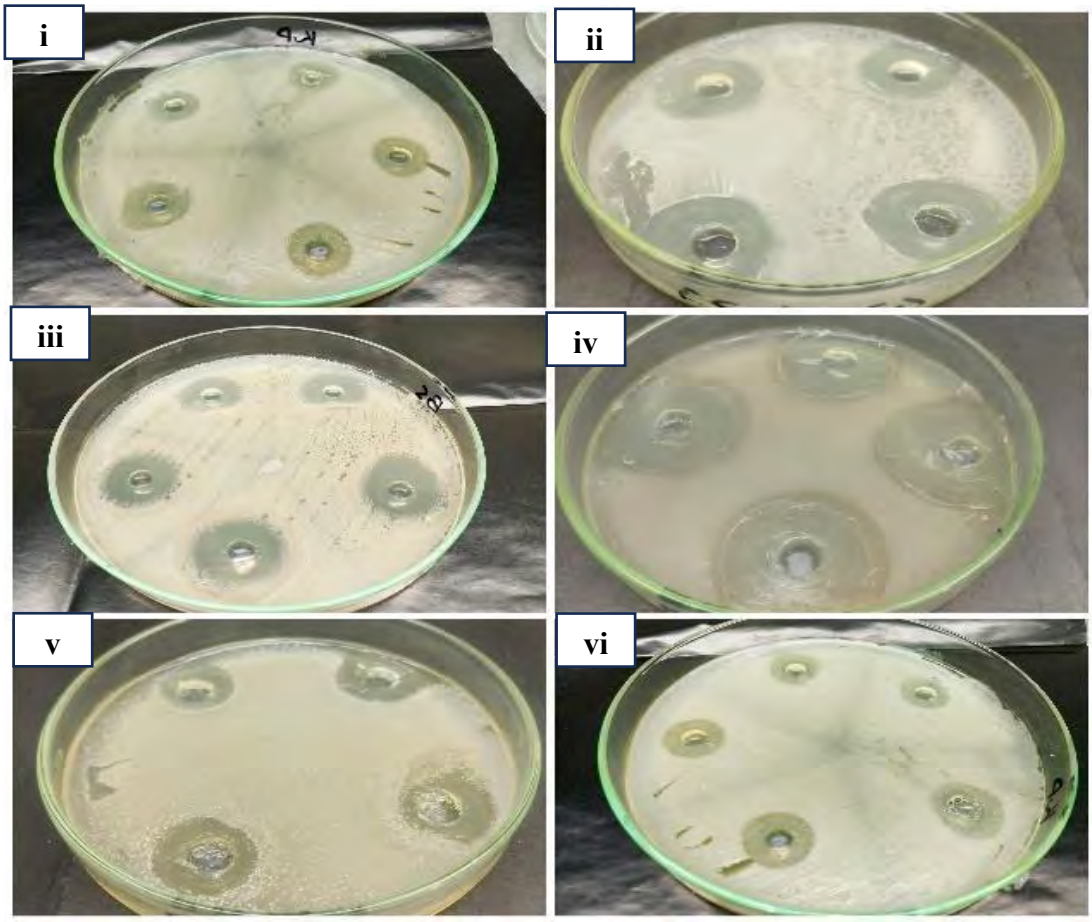


Figure 26 The antimicrobial metabolites of *G. candidum* forming zones of inhibition around the agar wells of (i) *Pseudomonas aeruginosa* ATCC 27853 (ii) *Salmonella enterica* ATCC 14028 (iii) *E. coli* ATCC 25922 (iv) *Bacillus subtilis* ATCC 5230, (v) *Staphylococcus aureus* ATCC 25923 (vi) *Clostridium sporogenes* ATCC 19404

Hence, the extracted metabolites (supernatant) from bacterial consortium and *G. candidum* proved to be antimicrobial in nature. The zones of inhibition formed by the bacterial metabolites depicted agood antimicrobial potential against specific pathogenic strains. Nevertheless, the metabolitesof *G. candidum* gave a much stronger antimicrobial activity by forming larger zones of inhibition against all tested pathogenic strains. Hence, *G. candidum* has a greater antimicrobial potential againsta variety of strains than that of the bacterial consortium.

Table 8 Diameter of zone of inhibition as a result of antimicrobial activity of bacterial consortium and *G. candidum*

Pathogen	Diameter of Zone of Inhibition	
	Bacterial Consortium	***G. candidum***
Pseudomonas aeruginosa ATCC 27853	11-18 mm	25-28 mm
Pseudomonas aeruginosa ATCC 9027	NIL	35-38 mm
Bacillus subtilis ATCC 5230	18-20 mm	38-41 mm
Bacillus subtilis ATCC 6633	NIL	37-40 mm
Staphylococcus aureus ATCC 25923	12-19 mm	27-28 mm
Staphylococcus aureus ATCC 6538	NIL	29-30 mm
Salmonella enterica ATCC 14028	14-15 mm	31-32 mm
E. coli ATCC 25922	11-13 mm	31-33 mm
Clostridium sporogenes ATCC 19404	NIL	22-24 mm
Candida albicans ATCC 10231	NIL	19-23m

4.21. Antibiotic Susceptibility (AST)

The bacterial strains in the consortium were **susceptible** to the antibiotics viz. Colistin Sulphate 10µg, Chloramphenicol 30µg, Gentamycin 10µg, Tetracycline 30µg, Clindamycin 2µg, and Neomycin 10µg, and **resistant** to Amoxycilin 10µg, Penicillin G 10µg, Cefepime 30µg, and Amikacin 30µg.

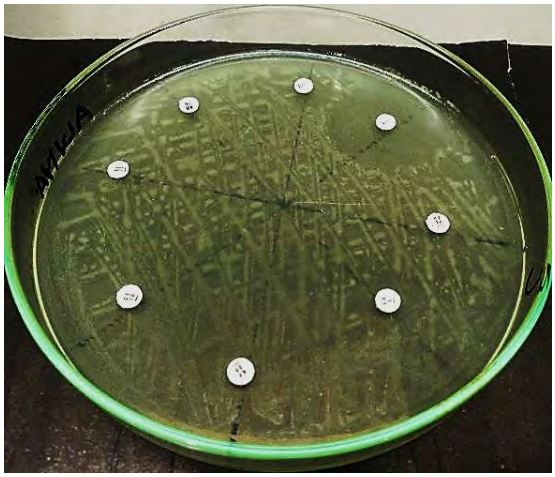


Figure 27 Zones of inhibition formed around specific antibiotic discs

Table 9 Diameter of zone of inhibition as a result of antibiotic action on the bacterial consortium

Antibiotic	Diameter of Zone of Inhibition
Amoxycilin 10 µg	No zone
Colistin sulphate 10 µg	22 mm
Clindamycin 2µg	10 mm
Penicillin G 10µg	No zone
Chloramphenicol 30µg	25 mm
Gentamycin 10µg	19 mm
Tetracycline 30µg	13 mm
Cefepime 30µg	No zone
Neomycin 10µg	14 mm
Amikacin 30µg	No zone

4.22. Antifungal Susceptibility (AFST)

The strain *G. candidum* was **susceptible** to the antifungals viz. Nystatin, Amphotericin B, Itraconazole, Voriconazole, and Fluconazole.

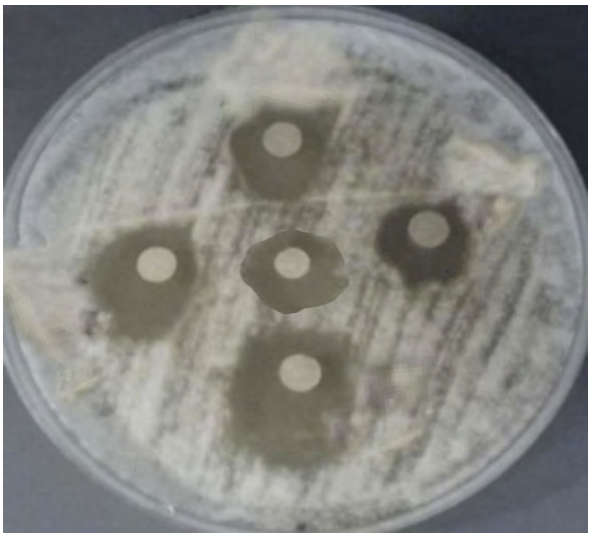


Figure 28 Zone of inhibition formed around each disc saturated with antifungal agent

Table 10 Diameter of zone of inhibition as a result of antifungal action on *G. candidum*

Antifungal	**Diameter of Zone of Inhibition**
Nystatin	20 mm
Amphotericin B	19 mm
Itraconazole	13 mm
Voriconazole	18 mm
Fluconazole	14 mm

CHAPTER 05
DISCUSSION

DISCUSSION

Nature has always provided man with matchless blessings for every need of life. From thetime a child is born to the period when his body matures, proper functioning must be guaranteed at all costs. For this purpose, breast milk has been declared by the nature as thecomplete nutrition for some early period of life (Lyons et al., 2020). However, over the course of evolution, the change in feeding practices have made infant formulas encompassa wide area of the industrial world. In the world where everything can be prepared by the use of science and technology, use of natural products is indispensable. This is because synthetic foods can never be designed to have balanced and sufficient contents as that in natural foods. Similarly, infant formulas are just a picture of the attempts made to mimic breast milk (Salminen et al., 2020). A better alternative is the use of animal source and plant- based milk products for feeding the young ones (Maryniak et al., 2022). Moreover, the introduction of probiotic bacterial and fungal strains is yet another factor that not only boosts the microbial perspective but also enhances nutritional value and shelf-life by carrying out fermentation of milk. However, keeping the quality of food intact is a challenging task, but the use of lyophilization technique extends the shelf-life of the product, and safety of microbial cultures up to years. Though it is humanly not possible to introduce all the reported probiotic in an infant formula, therefore researches have played their part in diagnosing the best probiotic strain to be supplemented in formula milk (Mercer & Arrieta, 2023).

The present study was designed to produce LABs and yeast fermented lyophilized infant formula milk products. Among all the reported probiotic microbes, *Streptococcus thermophilus* QAUSTN63, *Lactobacillus delbrueckii* subsp. *bulgaricus* QAULDN14, *Lactococcus lactis* QAULLNA8, and *Geotrichum candidum* QAUGC01 (PRJNA523005) were selected as indigenous gut microbes. The purpose of choosing these strains was their strong biochemical and probiotic potential as proved by their genomic and physiological characterization which they also had displayed in human gut as per the research conducted at Lab for Microbial Safety and Nutrition at QAU. The bacterial strains were used as the primary fermentation cultures while G. *candidum* was used as a secondary culture with the purpose of normalizing the milk pH and adding more benefits to the synthesized products. The bacterial strains *S. thermophilus* QAUSTN63 and *L. delbrueckii* QAULDN14, locally isolated from dahi, have been declared to possess biotherapeutic properties. Additionally, *L.s lactis* QAULLNA8 and *G. candidum* QAU GC01 (PRJNA523005)

are well-known for their role as cheese starters (Perkins et al., 2020). Several strains of *L. lactis* and *G. candidum* are now being used on a pilot scale worldwide. The combination of these four strains, to carry out the fermentation of milk, gives each type of milk both dahi and cheese like properties. Along with providing nutritional benefits, LABs rendered the milk products acidic after fermentation and acidic foods are not tolerated by infants. Therefore, another purpose of using *G. candidum* was used to neutralize the acidity of each milk product. The products were converted in powdered form so that they can be reconstituted into liquid, the most lightly digestible form, by the addition of water.

The products, after the set batch of fermentation, were lyophilized in order to store the nutrients and strains for a longer period of time. This was one of the steps towards long- term preservation of infant formula milk supplemented with probiotics. However, lyophilization can sometimes act as a stress factor for the microbial cultures surviving withina product, and they can lose their potential and can even reduce in number (Jameel, 2023).So, the next step was the determination of each strain's viability i.e. whether or not thestrain remains viable during the entire shelf life of the product, especially after lyophilization. The growth on agar plates showed the strain to be viable during different intervals during 3 months duration with an average of 10^8 CFUs per grams in each sample.For bacterial consortium, positive test results for lactose fermentation, curdling of milk, BSH activity, proteolytic activity, lipolytic activity, antimicrobial activity and antibiotic susceptibility have added to its use as potential probiotic with a pH range of 6-8 and optimum temperature range of 37^{o}C. Along with this, the NaCl and Bile salts tolerance makes it safe and beneficial for human consumption (Blandino, 2008). In addition, *G. candidum* showed positive test results for BSH activity, proteolytic activity, NaCL tolerance, Bile salts tolerance, antimicrobial activity and antifungal susceptibility with a pH range of 6-7 and optimum temperature range of 25^{o}C. Moreover, the nutritional profiling of each product showed highest crude protein in soy milk i.e. 9.4% and 27.01%, highest ash content i.e. 1.053% and 5.48%, and fat content i.e. 8.5% and 29.07%, total solids 19.44% and 96.82% in sheep milk in liquid and lyophilized product respectively. Moreover, the pH analysis of the products after the inoculation of *G. candidum* showed that the strain has increasedthe pH from 5 to 6. The pH of the lyophilized products also falls between the standard range i.e. 6-7. This has subsided the acidity of the product making it good for human use. Alongside this, the FTIR Spectral Analysis of each product before

and after lyophilizationwas also carried out. The IR absorbance spectrum before lyophilization for Soy milk has showed the medium appearance of secondary amines, and strong appearance of alkenes.

The FTIR absorbance spectrum of milk samples also added to their safe profile showing a range of useful and beneficial compounds. Before lyophilization, Soy milk and goat milk, has showed strong appearance of alkenes. For Skim milk, the absorbance spectrum has given appearance of alkanes and conjugated alkenes. For Cow milk, the absorbance spectrum has given medium appearance of secondary amines and alkenes. For Sheep milk, alkenes, esters, and primary alcohols were observed.

Similarly, The IR absorbance spectrum after lyophilization for Soy milk showed strong appearance of carboxylic acids, amine salts, aliphatic ketones, nitro compounds, sulphonylchloride, alkyl aryl ethers, and anhydrides. For Skim milk, strong appearance of nitro compounds, sulphonyl chlorides, and alkyl aryl ethers. For Cow milk, strong appearance of alcohols, amine salts, aldehydes, sulfoxides, and nitro compounds were observed. For Sheep milk, strong appearance of alcohols, amine salts, α, β-unsaturated esters, nitro compounds, sulfates, and aldehydes were noted. For goat milk, strong presence of alcohols, nitro compounds, alkyl aryl ethers, and alkenes was observed.

However, supplementation of only a few strains is never enough to perform the functions of the entire gut microbiota but it provides maximum benefits in the areas that need to be fulfilled to categorize a strain as safe and consumer-friendly. In short, the supplementation into the infant formula derived from mammalian milk and plant proteins has proven fruitful experimentally, and is expected to work biologically too providing immense favors, as the tummy of every tot matters.

CHAPTER 06

CONCLUSION

CONCLUSION

Breast milk has been declared as the matchless and indispensable source of food for an infant. However, where breastfeeding could not be continued, formula feeding came into play. In order to formulate a more natural and beneficial product, probiotic supplemented mammalian milk and plant-based milk were brought to limelight. These natural milk types proved fruitful for the formula fed lot by ensuring healthy gut microbiome. The bacterial strains viz. *S. thermophilus* QAUSTN63, *L. delbrueckii* QAULDN14, and *L. lactis* QAULLNA8; and the fungal strain *Geotrichum candidum* QAUGC01 (PRJNA523005) have been of great interest and concern due to the endless wonders they provide. The biochemical activity showed by these strains proved their potential for carrying out the life activities after activation in a suitable environment. Plus, the probiotic strength of the aforementioned strains remained intact as depicted by the positive results ensuring their viability and attributes stability after lyophilization. After the in vitro analysis, the selected strains were rendered a justifiable choice for supplementation in natural milk sources to produce nutritionally intact formula milk products i.e. Fermented Soy milk formula, Fermented Skim milk formula, Fermented Cow milk formula, Fermented Sheep milk formula, and Fermented Goat milk formula.

CHAPTER 07
FUTURE PROSPECTS

FUTURE PROSPECTS

The future holds a lot more, and some of the prospects are enlisted as follows:

- Other than the used probiotic strains, supplementation with other probiotics could be carried out to gradually expose the infant gut with majority of the probiotics.

- The cost can be justified by setting up a pilot scale production and lyophilization plant once for all. Also, wide production facilities can aid in producing large number of products in no time. This will help reduce the cost as the country will be having its own products.

- No doubt, the in vitro analysis of the probiotic viability and attribute stability have laid positive and reliable outcomes, but there is a need to carry out the in vivo analysis too. For this purpose, mice model (pups) with food allergy, food intolerance, and gut dysbiosis willbe the first step to check the strength and benefits of each product. It is believed that each product will have an advantageous outcome on each mice's guts.

- A comparison could be generated by taking into account the guts of mother milk fed, commercial formula fed, animal milk-based formula fed, and soy milk-based formula fed mice. However, it may be noted that the guts of mother milk fed mice will always be unmatched due to the countless benefits and significance of mother milk for infant gut. Yet, the synthesized products i.e. Fermented Soy milk formula, Fermented Skim milk formula, Fermented Cow milk formula, Fermented Sheep milk formula, and Fermented Goat milk formula can give much improved results for the mice gut health as compared to the artificially synthesized formulas.

- The products can act as nutraceuticals and biotherapeutics for adults, where the supplemented probiotics and the natural nutrients can help with gut dysbiosis by lowering cholesterol levels, degrading excessive fats in the body, curing inflammatory bowel disease, and improving gut health.

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ANNEX

MRS

- Glucose (Sigma-Aldrich) 18.5g
- Peptone (Biolife)10g
- Beef extract (Smart-Lab) 8g
- Yeast extract (Condalab) 4g
- Sodium acetate (Sigma) 3g
- Dipotassium phosphate (Samchun) 2g
- Ammonium citrate (Uni-chem) 2g
- Magnesium sulfate (AppliChem) 0.2 g
- Manganese sulfate 0.05g
- Tween 80 (Organics) 1ml (3 drops)
- Agar (BioWorld) 15g
- dH_2O 1000 ml

OGA

- Glucose (Sigma-Aldrich) 20g
- Yeast Extract (Condalab) 10g
- Agar (BioWorld) 15g
- dH_2O 1000 ml